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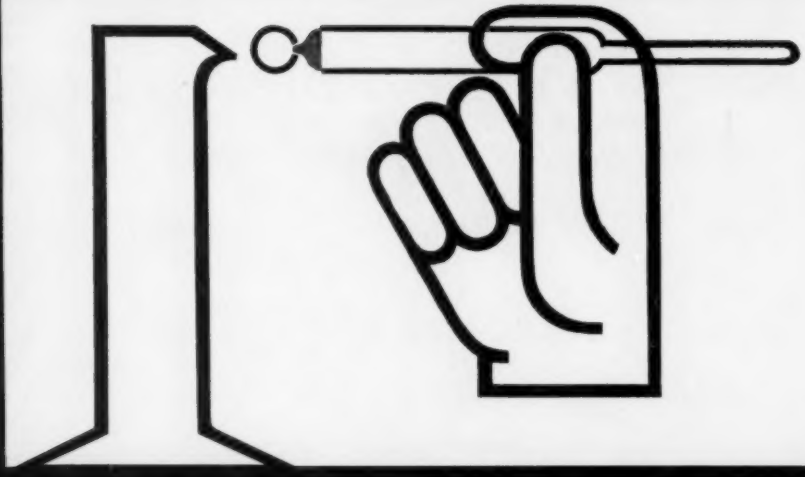

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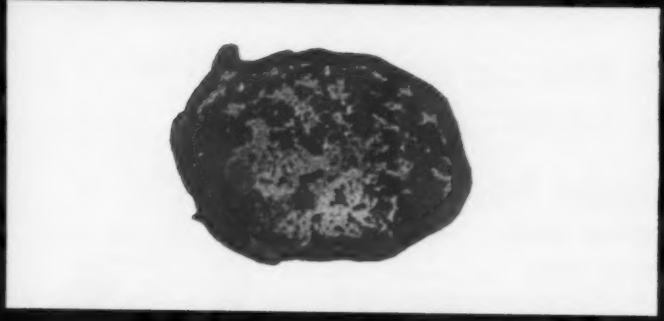

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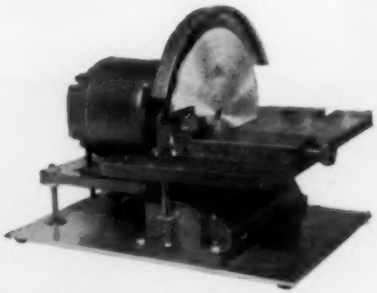

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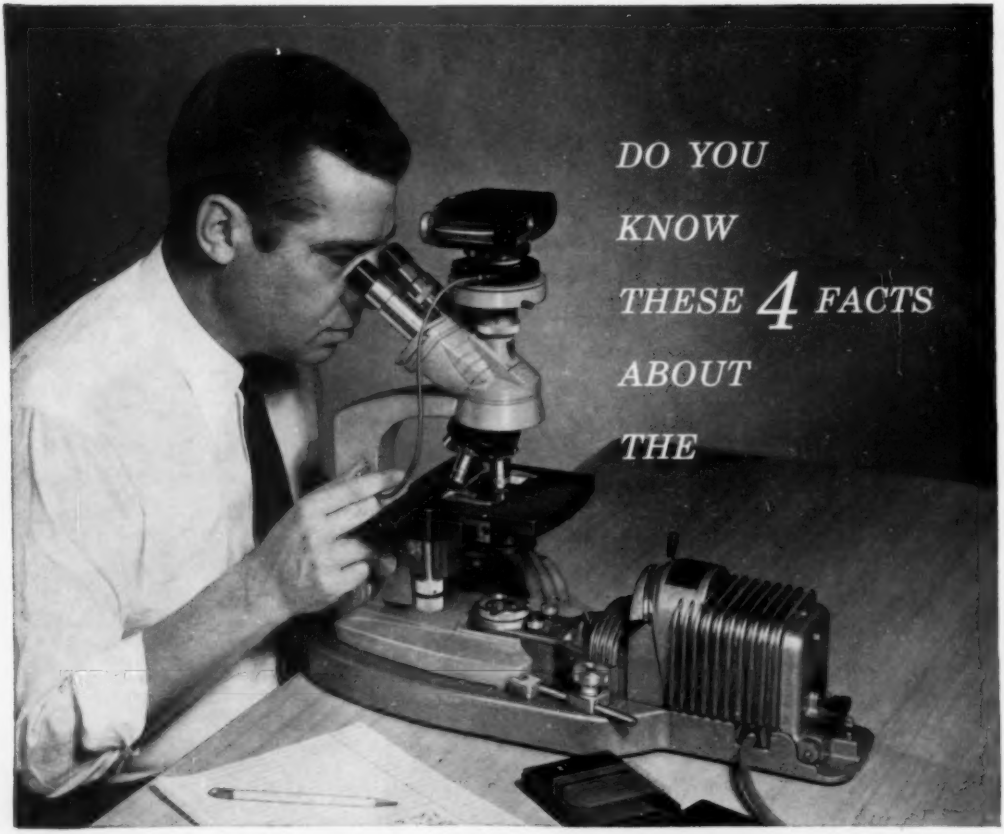

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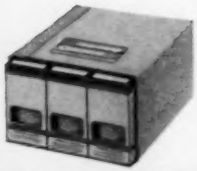

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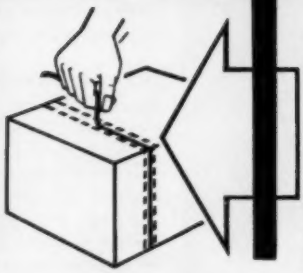

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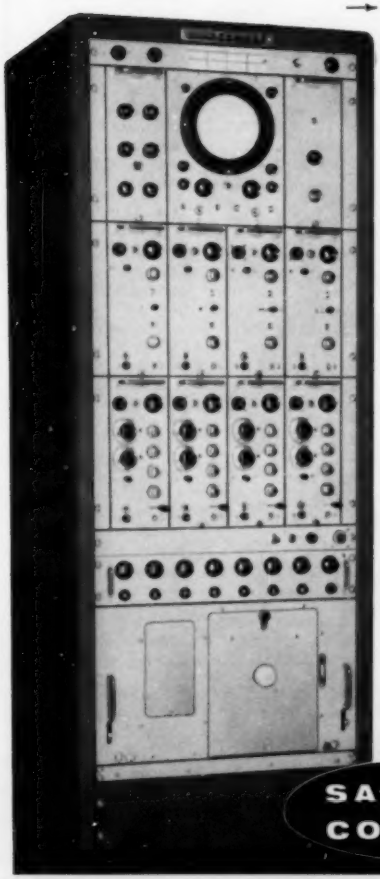

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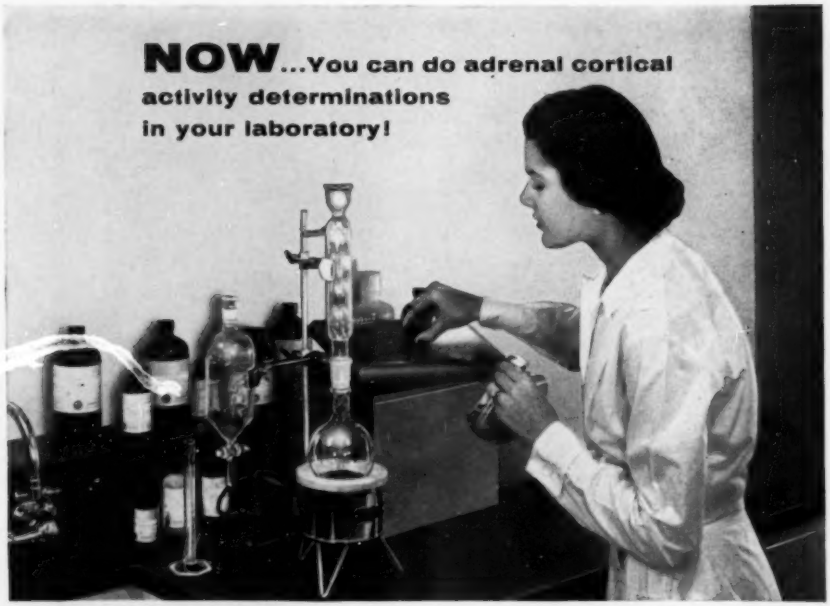

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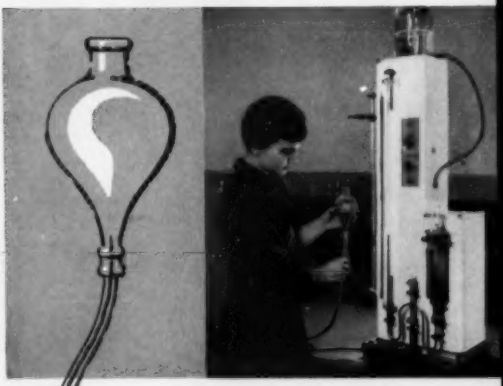

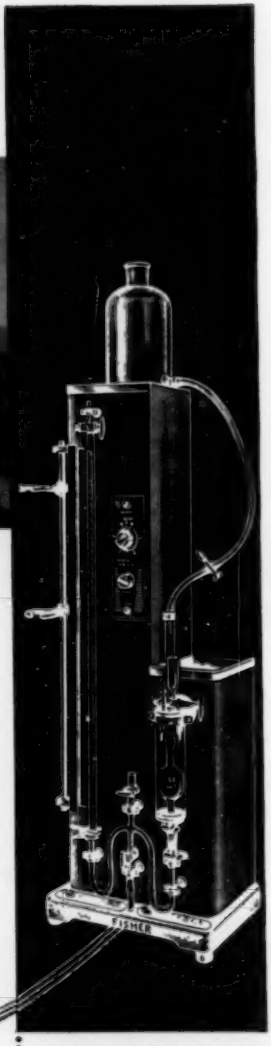

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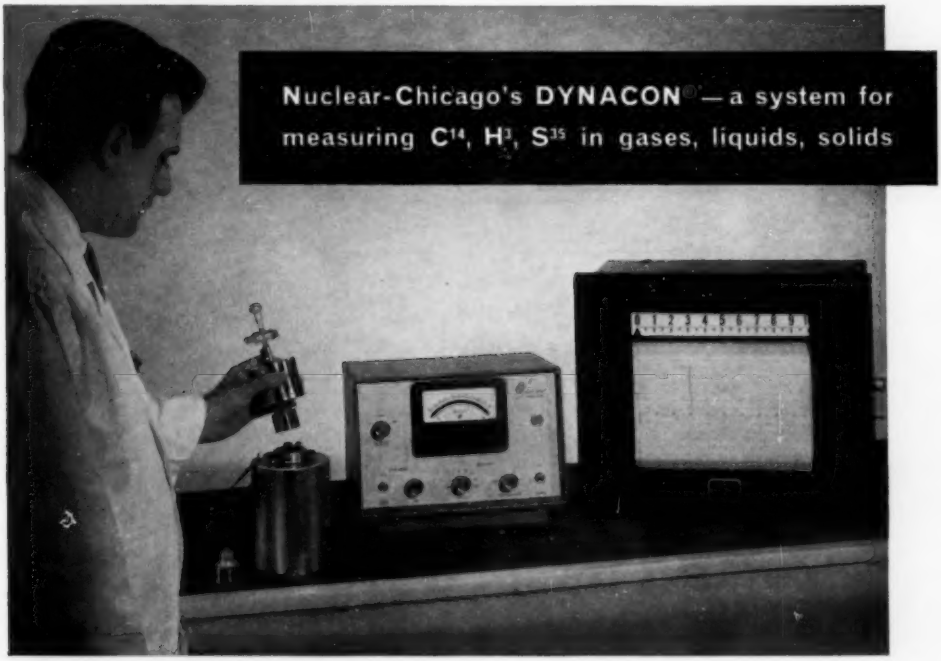

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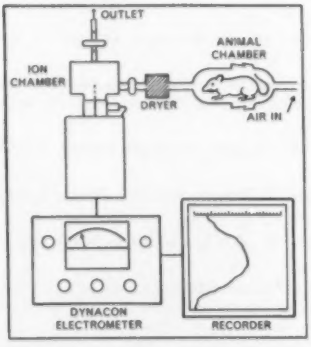

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The Natural History of Cancer. Dr. Leslie Foulds, Royal Cancer Hospital, London, England.

On the Etiology of Cancer. Dr. Michael B. Shimkin, National Cancer Institute, Bethesda, Md.

An Appraisal of Tumor-Virus Problems. Dr. Jacob Furth and Dr. Donald Metcalf, Children's Cancer Research Foundation, Boston, Mass.

A Critical Analysis of "Tumor Immunity." Dr. Morris K. Barrett, National Cancer Institute, Bethesda, Md.

Recent Studies on Pathophysiologic Mechanisms in Human Neoplastic Disease. Dr. Alfred Gellhorn, College of Physicians and Surgeons, University of Columbia, New York, N. Y.

Some Aspects of Virus-Induced Tumors. Dr. R. J. C. Harris, Institute of Cancer Research, London, England.

Radiation-Induced Leukemia in Man, With Particular Reference to the Dose-Response Relationship. Dr. W. M. Court-Brown, Medical Research Council, Western General Hospital, Edinburgh, Scotland.

The National Cancer Chemotherapy Program. Dr. K. M. Endicott, National Cancer Institute, Bethesda, Md.

Clinical Investigations in Cancer Chemotherapy. Dr. Charles G. Zubrod, National Cancer Institute, Bethesda, Md.

Reflections From Cancer Chemotherapy Screening Studies. Dr. C. Chester Stock, Sloan-Kettering Institute, New York, N. Y.

The Immunologic Theory of Cancer: Some Implications in Human Pathology. Dr. H. N. Green, Director of Cancer Research, School of Medicine, University of Leeds, Leeds, England.

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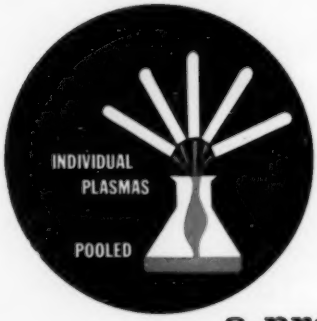

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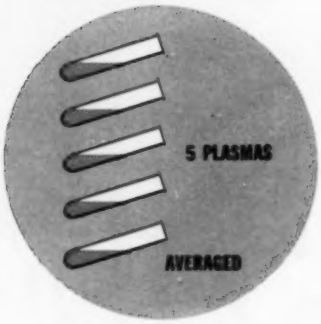

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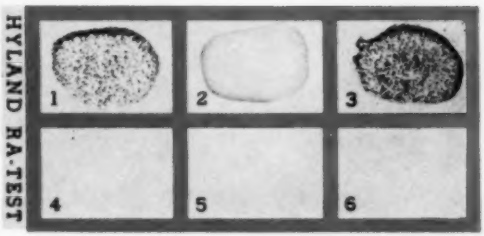

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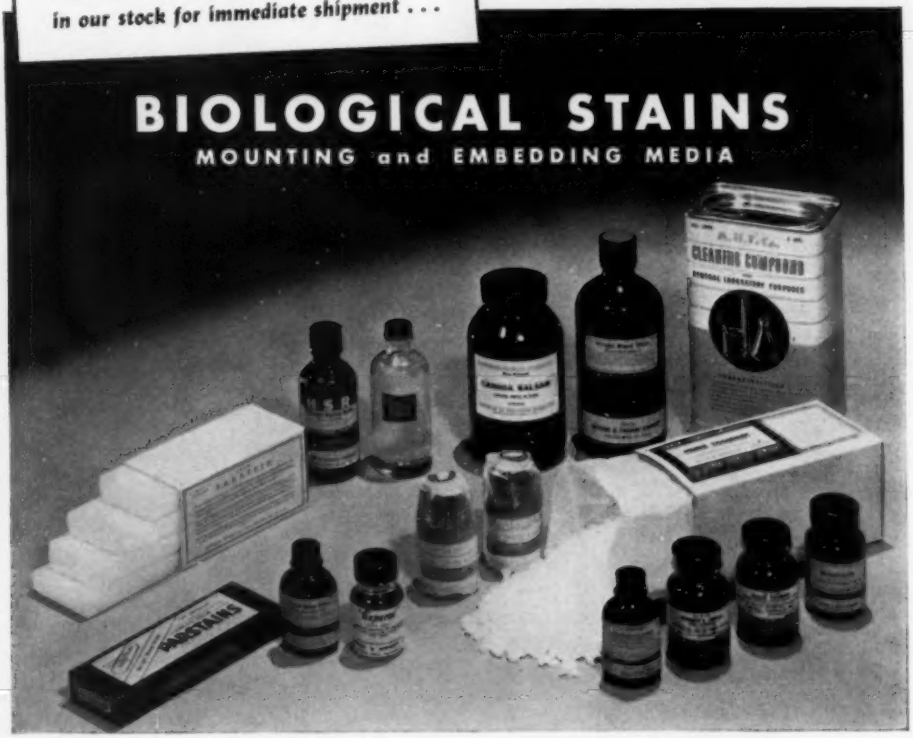

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The Journal of Laboratory and Clinical Medicine

VOL. 52 JULY, 1958 No. 1

CLINICAL AND EXPERIMENTAL

THE DETERMINATION OF ULTRAFILTRABLE CALCIUM IN A VARIETY OF CLINICAL CONDITIONS

ANANDA S. PRASAD, M.B., B.S., PH.D., AND EDMUND B. FLINK, M.D., PH.D.
MINNEAPOLIS, MINN.

THE lack of a convenient method for ultrafiltration has been responsible for the paucity of clinical investigation of ultrafiltrable calcium, which in turn is an approximation of ionic calcium. The method for the determination of ultrafiltrable calcium as reported by us previously[1] is convenient and useful for this purpose. The results of the determination of ultrafiltrable calcium in different clinical disorders in a large group of patients will be reported.

METHODS

Blood was collected under oil. Total and ultrafiltrable calcium was determined by Clark and Collip's modification of Kramer and Tisdall's method.[2] Ultrafiltration was done by a method reported by us previously.[1] When acidosis or alkalosis was present, the centrifuge tube was flushed with proper CO_2 and O_2 gas mixtures as described previously. In these cases the serum pH was determined before and after ultrafiltration by means of a research model Cambridge pH meter using anaerobic electrodes at 37 C.° Flushing of the centrifuge tube was considered unnecessary when there was no clinical evidence for acid base disturbance (see Discussion). Total protein was determined by the biuret method.[3] Albumin and globulin ratio was also determined.[4, 5] Phosphorus was determined by the method of Fiske and Subbarow.[6] The normal values for our laboratory for total and ultrafiltrable calcium are as follows: total calcium, 9.84 mg. per cent (S.D. ±0.46); ultrafiltrable calcium, 5.24 mg. per cent (S.D. ±0.214); and percentage of ultrafiltrable calcium, 53.3 per cent (S.D. ±2.48).[1] A difference greater than 3 times the standard deviation from the normal mean value was used to decide if the particular values were abnormal.

From the Medical Service, Veterans Administration Hospital, and the Department of Internal Medicine, University of Minnesota, Minneapolis, Minn.

Presented in part at the American Federation for Clinical Research, Chicago, Oct. 31, 1957 (Clin. Res. Proc. 5: 301, 1957).

Received for publication Dec. 6, 1957.

RESULTS

The ultrafiltrable serum calcium in patients with hyperparathyroidism, hypoparathyroidism, and metabolic acidosis with and without hyperphosphatemia has been plotted against the total serum calcium in Fig. 1. Eight patients with hyperparathyroidism were investigated. Elevated values for both

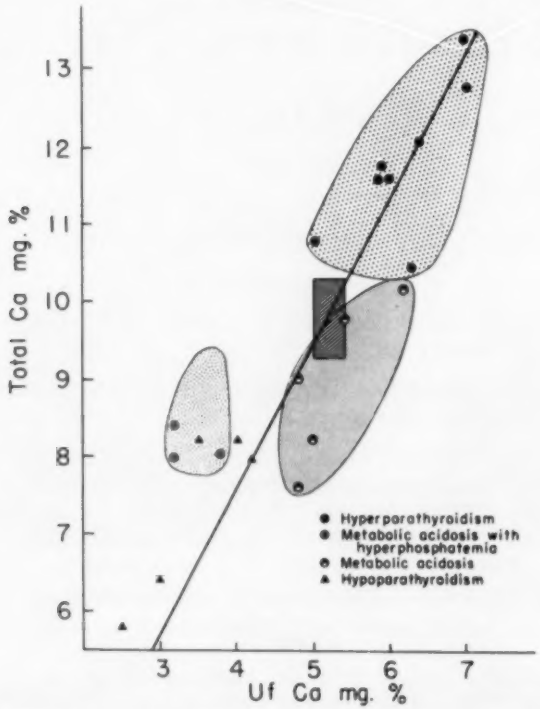

Fig. 1.—Central rectangular area has been drawn to represent normal mean values for total and ultrafiltrable calcium with one standard deviation range. The straight line has been drawn assuming the normal mean percentage of ultrafiltrable calcium to be 53 per cent of the total calcium.

total and ultrafiltrable calcium were found in 6 cases. One patient, A. B., had a normal total calcium but an elevated ultrafiltrable calcium. This was a 56-year-old white woman whose illness dated back to 1953. In 1955, a diagnosis of disseminated lupus erythematosus and Sjögren's syndrome was made. Because of her protein abnormality a total and ultrafiltrable calcium were determined and found to be 10.4 and 6.8 mg. per cent, respectively. This suggested hyperparathyroidism; accordingly, further studies were carried out and later a parathyroid adenoma was removed. Following this the dysphagia and dryness of the mouth and eyes disappeared. A few weeks postoperatively the total and ultrafiltrable calcium were 8.2 and 4.0 mg. per cent, respectively.

Another patient, C. O., was uremic and had a history of renal calculi. The total and ultrafiltrable calcium were 10.8 and 5.0 mg. per cent, respectively. Blood urea nitrogen was 150 mg. per cent. On forced fluid therapy the blood urea nitrogen decreased to 19 mg. per cent and the total calcium and phosphorus were 13.1 and 2.6 mg. per cent, respectively. At this time parathyroidectomy was considered necessary and an adenoma was removed.

Five patients with hypoparathyroidism had decreased ultrafiltrable calcium ranging from 2.5 to 4.2 mg. per cent with total calcium ranging from 5.8 to 8.2 mg. per cent.

The results in patients with metabolic acidosis and associated hyperphosphatemia are also presented here. In all these patients the ultrafiltrable calcium was markedly decreased. In this group the serum phosphorus ranged from 9.2 to 12.0 mg. per cent. The initial serum pH ranged from 7.20 to 7.27, and the pH at the end of ultrafiltration ranged from 7.30 to 7.60. The blood urea nitrogen ranged from 87 to 160 mg. per cent.

In 5 other cases of metabolic acidosis presented here, the range for blood urea nitrogen was 62 to 133 mg. per cent and serum phosphorus ranged from 4.3 to 7.8 mg. per cent. The initial serum pH range was 7.22 to 7.40 and the pH after ultrafiltration ranged from 7.42 to 7.63. In these cases the ultrafiltrable calcium was normal except in one where it was elevated. This patient had carbon tetrachloride poisoning. He had a normal total calcium but the ultrafiltrable calcium was increased. Although the serum phosphorus was somewhat increased in this group also, it was not quite so marked as in the previous one.

Two cases of metabolic alkalosis, not shown in this figure, had normal total and ultrafiltrable calcium. One patient, C. H., had duodenal ulcer with pyloric obstruction. He developed severe vomiting, metabolic alkalosis and renal failure. The blood urea nitrogen was 93 mg. per cent and serum phosphorus was 12.6 mg. per cent. The initial serum pH was 7.47 and at the end of ultrafiltration it was 7.77. He had manifest tetany on admission. The total and ultrafiltrable calcium were 9.2 and 3.6 mg. per cent, respectively. A detailed report of this case has appeared elsewhere.[18]

TABLE I. ELEVATED ULTRAFILTRABLE CALCIUM IN CASES OTHER THAN HYPERPARATHYROIDISM

PATIENT	TOTAL Ca (MG./100 ML. SERUM)	UF. Ca (MG./100 ML. UF.)	PER CENT OF UF. Ca	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	GLOBULIN (GM. %)	MISCELLANEOUS DATA
O.	16.0	6.6	41.2				Metastatic carcinoma of breast
N. H.	13.6	6.6	48.5	5.4	2.7	2.7	Metastatic carcinoma of breast
A. C.	14.6	7.8	53.4	6.4	3.6	2.8	Metastatic carcinoma of rectum; phosphorus 3.2 mg. per cent
F.	15.3	6.6	43.1	15.3	4.6	10.7	Multiple myeloma
A. A.	9.8	6.0	61.2	10.0	3.2	6.8	Multiple myeloma
J. K.	12.4	7.0	56.4	6.8	3.4	3.4	Multiple myeloma
B. H.	13.4	6.4	47.8	8.0	3.0	5.0	Multiple myeloma
E. H.	10.4	5.9	56.7	13.8	3.3	10.5	Multiple myeloma
L. B.	11.6	6.8	58.6	12.2	2.9	9.3	Multiple myeloma

The patients with an elevated ultrafiltrable calcium in conditions other than hyperparathyroidism and metabolic acidosis without hyperphosphatemia are presented in Table I. Out of 15 cases of multiple myeloma, elevated ultrafiltrable calcium was found in 6 instances. In two cases, A. A. and E. H., increased ultrafiltrable calcium was associated with normal total calcium. Both of these cases had marked protein abnormalities. The other patients with multiple myeloma had normal ultrafiltrable calcium (see Table III). Three cases of metastatic carcinoma had both total and ultrafiltrable calcium increased.

Table II shows the results where decreased values for ultrafiltrable calcium were found in cases other than hypoparathyroidism, hyperphosphatemia, and muscular dystrophy. These patients were chronically ill and debilitated. Four patients with thyrotoxicosis were studied. Two of these had low ultrafiltrable calcium. Out of 19 cases of cirrhosis of the liver, 3 had decreased ultrafiltrable calcium. Three cases of idiopathic steatorrhea were studied, one of which had decreased ultrafiltrable calcium. A case of idiopathic hypoalbuminemia which had decreased ultrafiltrable calcium is also included in this table.

TABLE II. DECREASED ULTRAFILTRABLE CALCIUM IN CASES OTHER THAN HYPOPARATHYROIDISM, HYPERPHOSPHATEMIA, AND MUSCULAR DYSTROPHY

PATIENT	TOTAL Ca (MG./100 ML. SERUM)	UF. Ca (MG./100 ML. UF.)	PER CENT OF UF. Ca	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	GLOBULIN (GM. %)	MISCELLANEOUS DATA
J. H.	10.4	4.2	40.4	7.5	3.5	4.0	Hyperthyroidism
J. E.	9.0	4.3	47.8	5.3	1.5	3.8	Hyperthyroidism
N. B.	8.8	3.5	39.8	4.4	1.9	2.5	Idiopathic hypoalbuminemia
A. J.	7.5	4.2	56.0	4.5	2.2	2.3	Cirrhosis of liver
O. T.	7.6	4.4	57.9	3.7	2.7	1.0	Cirrhosis of liver
D. W.	8.8	4.0	45.4	6.1	1.7	4.4	Cirrhosis of liver
P. L.	6.2	3.6	58.0	6.3			Idiopathic steatorrhea
T. G.	7.8	4.0	51.3	4.9	1.6	3.3	Carcinoma of stomach

The data obtained in miscellaneous conditions are shown in Table III. Out of 4 cases of sarcoidosis, one had increased total calcium, but the ultrafiltrable calcium was normal in all the cases. In 27 cases of hypoalbuminemia the majority had normal ultrafiltrable calcium. A high percentage of the ultrafiltrable calcium was found in 3 instances associated with marked hypoalbuminemia. Seven patients with renal calculi of unknown etiology, 2 patients with macroglobulinemia, one with hypogammaglobulinemia, one with Paget's disease, and one with myasthenia gravis had normal total and ultrafiltrable calcium. Seven cases of muscular dystrophy and one case of dermatomyositis had decreased ultrafiltrable calcium. In other cases of muscular dystrophy which were investigated, the ultrafiltrable calcium was within the normal range.

The cases with decreased total calcium but normal ultrafiltrable calcium are presented in Table IV. All of these had low serum albumin.

TABLE III

CONDITIONS	NO.	CALCIUM			CALCIUM				PROTEINS				
					NUMBER INCREASED		NUMBER DECREASED				NO. OF CASES*		
		RANGE TOTAL	RANGE UF.	RANGE % UF.	TOTAL	UF.	TOTAL	UF.	RANGE ALBUMIN	RANGE GLOBULIN	T.P. < 5	A < 3	G > 4
Sarcoidosis	4	10.0-11.6	4.6-5.4	46.0-50.0	1				2.2-3.3	2.4- 6.6		1	2
Hypoalbuminemia	27	6.0-10.5	3.5-5.6	39.8-80.0			11	6	1.0-2.8	1.0- 4.4	15	27	2
Multiple myeloma	15	9.8-15.3	5.0-7.0	43.1-62.5	4	6			2.3-4.6	2.4-14.2		8	12
Cirrhosis of liver	19	7.5-10.4	4.0-5.5	46.1-70.5			4	3	1.0-3.7	1.0- 8.0	3	17	12
Collagen diseases	4	8.8-10.2	4.2-4.8	42.8-52.0				1	1.7-3.7	2.9- 6.3		2	2
Hyperthyroidism	4	9.0-10.6	4.2-5.0	40.4-47.8				2	1.5-3.5	3.8- 4.6		1	1
Muscular dystrophy	21	9.4-10.8	4.2-5.8	40.0-59.6				7	2.7-4.7	2.4- 4.0		1	

Calcium is expressed in milligrams per cent. Proteins are expressed in grams per cent.
*T.P. = total protein; A = Albumin; G = Globulin.

A comparison of values of ultrafiltrable calcium when it was determined directly from that obtained by McLean and Hasting's nomogram[7] and Ziesler's mathematical formula[8] is shown in Table V. In the cases reported here, poor agreement between the actual values and the derived ones was found in the abnormal conditions.

TABLE IV. CASES HAVING LOW TOTAL CALCIUM BUT NORMAL ULTRAFILTRABLE CALCIUM

PATIENT	TOTAL Ca (MG./100 ML. SERUM)	UF. Ca (MG./100 ML. UF.)	PER CENT OF UF. Ca	TOTAL PROTEIN (GM. %)	ALBUMIN (GM. %)	GLOBULIN (GM. %)	MISCELLANEOUS DATA
J. M.	8.2	4.6	56.1	6.0	1.9	4.1	Chronic osteomyelitis; CA of ampulla of Vater
A. R.	8.2	4.8	58.5	6.3	1.8	4.5	Cirrhosis of liver
A. T.	7.8	5.5	70.5	5.7	1.6	4.1	Cirrhosis of liver
A. A.	8.4	4.9	58.3	4.2	1.9	2.3	Idiopathic steatorrhea
S. S.	8.2	4.8	58.5	5.7	2.6	3.1	Idiopathic steatorrhea
E. L.	8.0	5.6	70.0	3.7	1.1	2.6	Nephrotic syndrome
R. B.	6.0	4.8	80.0	5.9	1.9	4.0	Nephrotic syndrome
M. G.	8.2	4.6	56.1	4.2	2.0	2.2	Nephrotic syndrome
A. W.	8.0	5.2	65.0	4.7	1.2	3.5	Arteriosclerotic heart disease

TABLE V. COMPARISON OF DIRECT AND INDIRECT DETERMINATION OF ULTRAFILTRABLE CALCIUM

PATIENT	TOTAL CALCIUM (MG. %)	ULTRAFILTRABLE CALCIUM (MG. %)			MISCELLANEOUS DATA
		DIRECT METHOD	USING FORMULA*	USING NOMOGRAM	
F. J.	10.2	4.8	4.4	4.5	Normal
O. H.	9.6	5.0	4.7	4.6	Normal
P. C.	9.6	5.2	4.0	4.0	Renal calculi
M. L.	10.0	5.3	3.9	4.0	Renal calculi
D. S.	11.0	5.2	4.3	4.5	Hyperthyroidism
J. M.	9.8	5.4	4.2	4.2	Cirrhosis of liver
B. A.	13.4	7.2	5.7	6.0	Hyperparathyroidism
H. V.	9.4	5.4	4.0	4.0	Blastomycosis
O. K.	9.3	5.1	3.7	3.6	Duodenal ulcer

*Ziesler's Formula: $\text{Ionic Calcium} = \dfrac{6\ \text{Total Calcium} - \frac{\text{Total Protein}}{3}}{6 + \text{Total Protein}}$

DISCUSSION

Several workers[7, 9, 10] believed that the level of the ultrafiltrable calcium is under the influence of the parathyroid glands. Recently Hopkins and associates[11, 12] have reported that all but one of a group of 9 patients with hyperparathyroidism had ultrafiltrable calcium values distinctly higher than their normal series. Their 8 patients with hypoparathyroidism had values distinctly lower than their normal individuals. Patients with diseases of bone having elevated total serum calcium values had elevated ultrafiltrable calcium. Their results differ in one respect from ours. The percentage of ultrafiltrable calcium is consistently greater in their cases than ours. They saturated the serum with 5 per cent CO_2 and O_2 prior to ultrafiltration in every case and reported that 65 to 75 per cent of the total calcium was ultrafiltrable in the normal persons. We have tried to explain this difference by the fact that the pH is

initially lowered below normal by handling the serum in this way.[1] Recently Toribara, Terepka, and Dewey[13] have reported that for healthy human subjects the range of ultrafiltrable calcium is 60 to 70 per cent of the total. Table VI shows the results obtained by using Toribara's techniques in the normal individuals. The blood was collected under oil and the tubes containing serum in the cellophane casing were flushed with 5 per cent CO_2 and O_2 for 30 to 50 seconds instead of 3 minutes as done by Toribara and co-workers.[13] In the normal persons the mean ultrafiltrable calcium was 5.24 mg. per cent, the mean percentage was 54.3 per cent of the total serum calcium, and the range of pH of the residual serum was 7.40 to 7.75. Table VII shows that diffusion

TABLE VI. ULTRAFILTRABLE CALCIUM IN NORMAL SERA

PATIENT	TOTAL Ca (MG. %)	UF. Ca (MG. %)	PER CENT UF. Ca	pH OF SERUM AT END OF UF.	TOTAL PROTEIN (GM. %)
H. S.	9.3	5.0	53.7	7.60	6.8
L. D.	10.3	5.4	52.3	7.59	7.3
H. V.	9.4	5.4	57.4	7.75	7.5
R. B.	9.5	5.2	54.7	7.64	6.7
A. W.	10.0	5.5	55.0	7.55	8.0
O. K.	9.3	5.1	54.8	7.44	8.1
		5.2	55.0		
E. W.	9.2	5.1	55.4	7.57	
M. V.	10.0	5.3	53.0	7.60	7.2
V. S.	9.5	5.5	57.6	7.70	7.5
P.	10.0	5.0	50.0	7.45	
W.	9.8	5.2	53.0	7.40	
Mean	9.57	5.24	54.3% pH	range 7.40 to 7.75	

Ultrafiltration was done by using Toribara's tubes. Fifteen milliliters of serum was centrifuged for 6 hours. The tubes were flushed for 30 to 50 seconds with 5 per cent CO_2 and 95 per cent O_2 prior to ultrafiltration. Blood was collected and serum separated under oil. pH was determined by means of a research model Cambridge pH meter using anaerobic electrodes at 37° C. The percentage was calculated without correction for the volume occupied by solids of sera.

TABLE VII

SAMPLES	PLASMA COLLECTED UNDER OIL		PLASMA EXPOSED TO AIR	
	pH BEFORE EQUILIBRATION	pH AFTER EQUILIBRATION	pH BEFORE EQUILIBRATION	pH AFTER EQUILIBRATION
1	7.40	7.28	7.70	7.32
		7.28		7.30
2	7.45	7.29	7.81	7.30
		7.31		7.30
3	7.35	7.22	7.60	7.25
		7.25		7.25
4	7.36	7.27	7.60	7.26
		7.27		7.26

Five per cent CO_2 and 95 per cent O_2 were allowed to diffuse for 3 to 4 minutes outside the cellophane casing containing plasma in ultrafiltration tubes as described by Toribara.

of 5 per cent CO_2 in O_2 outside the cellophane casing containing serum for 3 to 4 minutes in Toribara's tubes results in an initial decrease in the serum pH level. We believe this accounts for their higher values of ultrafiltrable calcium in normal sera. Furthermore, in Toribara's paper (Table I),[13] the pH of the residual serum in all instances was lower than the respective initial samples. As the temperature is lowered further, more CO_2 is dissolved in the serum and a further decrease in pH may be expected. The decreased serum

pH initially would explain their increased values of ultrafiltrable calcium at lower temperatures. The biologic methods as used by McLean and Hastings[14] and the indirect evidence obtained by in vitro calcification of rachitic rat cartilage in normal and pathologic human sera as reported by Yendt, Connor, and Howard[15] support the idea that in normal individuals the extracellular fluid calcium (ultrafiltrable calcium) concentration is less than that reported by Hopkins and associates[11, 12] and Toribara and co-workers.[13]

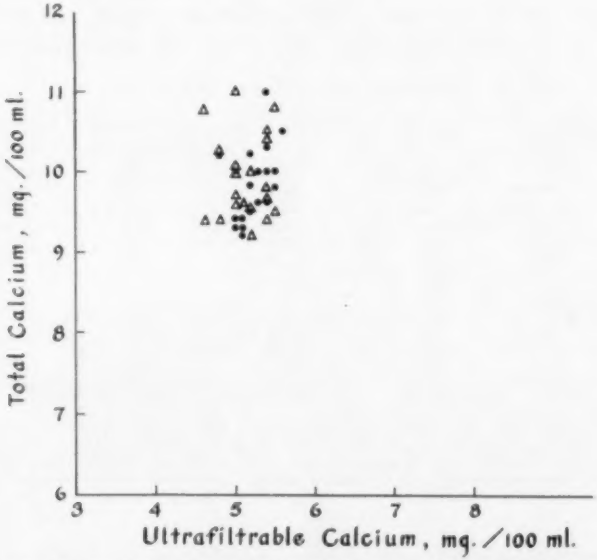

Fig. 2.—Group I (•), serum handled under oil. pH was controlled by allowing 5 per cent CO_2 in O_2 to diffuse outside the casing containing serum. Group II (△), serum handled under oil but 5 per cent CO_2 not diffused outside the casing containing serum.

As shown in Fig. 2, it is evident that the values for ultrafiltrable calcium obtained in Group I and II overlap each other and, statistically, are not significantly different. In both the groups the blood was collected under oil and the subjects were normal individuals. In Group I, 5 per cent CO_2 in O_2 was allowed to diffuse outside the casing containing the serum, as described previously[1] but in Group II this procedure was not carried out. In the latter group, the pH of the serum at the end of ultrafiltration ranges from 7.65 to 7.85. This indicates that separation of serum under oil and transfer to the casing without much delay for ultrafiltration is adequate in cases not associated with acid-base disturbance. However, we believe that if the serum is not separated under oil, the pH may change considerably more and this may result in lower values for ultrafiltrable calcium.

The results reported here indicate that the ultrafiltrable calcium is usually elevated in hyperparathyroidism and is reduced in hypoparathyroidism. In

one patient, C. O., with hyperparathyroidism and uremia, the total and ultrafiltrable calcium were normal. Although a serum phosphorus was not done at the time the ultrafiltrable calcium was determined, one may presume that hyperphosphatemia due to renal failure was present. The normal level of ultrafiltrable calcium in the presence of hyperphosphatemia may possibly suggest hyperparathyroidism, but this cannot be stated categorically from this one instance. Subsequently with proper fluid therapy the blood urea nitrogen returned to normal level and hypercalcemia and hypophosphatemia became apparent. Later a parathyroid adenoma was removed.

In some cases of marked hypoalbuminemia a normal absolute value but an increased percentage of ultrafiltrable calcium was noted. In one patient the total calcium was 6.0 mg. per cent. In spite of the low total calcium the absence of signs of tetany was explained when the ultrafiltrable calcium was determined and found to be 4.8 mg. per cent. Another patient with hypoalbuminemia had a low total serum calcium (7.8 mg. per cent) but the ultrafiltrable calcium was 5.5 mg. per cent which explained the absence of any symptoms or signs referable to hypocalcemia. Thus, a low total calcium may be associated with normal ultrafiltrable calcium. It appears that a decreased total calcium level in cases of cirrhosis or nephrotic syndrome or other states associated with hypoalbuminemia are accounted for by a decrease in the protein-bound calcium.

In acidosis associated with hyperphosphatemia, a decreased ultrafiltrable calcium level was found. The cardiac effects in uremia with hyperphosphatemia and hyperkalemia may be related to concomitant decrease in the level of ionic calcium. Recently ventricular fibrillation and cardiac arrhythmias occurring after hypercapnia in dogs have been attributed to hyperkalemia in the presence of decreased ultrafiltrable calcium concentration.[16, 17] The manner in which an increased concentration of phosphate results in a decrease in the ultrafiltrable calcium is not clear at present. It is possible that some nonfiltrable calcium phosphate compound is formed and the probability of such an event with these high concentrations of phosphate seems likely.

SUMMARY

Serum ultrafiltrable calcium was determined in a large series of patients. Elevated values were found in hyperparathyroidism, in some cases of multiple myeloma and metastatic cancer, and in acidosis not associated with marked hyperphosphatemia. Decreased values were found in hypoparathyroidism, in cases associated with hyperphosphatemia and in a few other debilitated states. In some cases of muscular dystrophy a lowered percentage of ultrafiltrable calcium was observed, the exact significance of which remains unknown at present. In cases of metabolic acidosis with hyperphosphatemia and hyperkalemia, the cardiac effects may be related to hyperkalemia and concomitant decrease in the level of ionic calcium.

Determination of the ultrafiltrable calcium has led to the correct diagnosis or to better understanding of the status of serum calcium in some of the cases reported here.

A normal total calcium may be associated with abnormal ultrafiltrable calcium, particularly in cases with abnormal proteins, acidosis, alkalosis, and hyperphosphatemia.

A comparison of determined ultrafiltrable calcium with that calculated from the McLean and Hastings nomogram or Ziesler's mathematical formula showed poor agreement in abnormal cases, which were investigated in this study.

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SOME OBSERVATIONS ON PATIENTS WITH HYPERCALCEMIA EXEMPLIFYING PROBLEMS IN DIFFERENTIAL DIAGNOSIS, ESPECIALLY IN HYPERPARATHYROIDISM

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IT HAS been approximately 30 years since hypercalcemia was first recognized in patients with hyperparathyroidism[1, 2] and in those given large amounts of vitamin D.[3] Since these early observations, it has become evident that an increase in concentration of serum calcium may occur in a variety of clinical disorders. As hypercalcemia per se is injurious,[4] or even lethal,[5] to the individual, recognition of the cause and prompt correction is important. Often, the diagnostic problem is one of differentiating hyperparathyroidism, which requires surgical intervention, from other causes of hypercalcemia. The etiologic possibilities that must be considered are listed in Table I. Each of these

TABLE I. CAUSES OF HYPERCALCEMIA OTHER THAN HYPERPARATHYROIDISM*

Laboratory error
Neoplastic disease (with or without demonstrable skeletal metastases) lymphoma; leukemia; multiple myeloma; carcinoma of lung, kidney, G.I. tract, ovary, breast, etc.
Sarcoidosis
Hypervitaminosis D
Milk-alkali syndrome
Hyperthyroidism
Idiopathic hypercalcemia of infants
Acute skeletal atrophy of disuse

*Hypercalcemia may occur in other situations, e.g., in acute adrenal insufficiency and in citrate intoxication, but, if present under such circumstances, seldom presents a problem in differential diagnosis.

maladies may be accompanied by hypercalcemia, and, at times, hypophosphatemia. Because the history, physical examination, and usual clinical studies may not afford sufficient evidence for precise diagnosis, additional aids have been sought. Recently the response to intravenous calcium,[6-10] measurement of tubular reabsorption of phosphorus,[10-13] and the effect of cortisone or related steroids,[14-15] have been used in the study of patients with hyperparathyroidism and, by some, have been deemed of value in recognizing the presence of this

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disorder. Oddly enough, little or no attention has been given to the limitations of these procedures in distinguishing one cause of hypercalcemia from another. Observations herein reported indicate that, although these studies provide informative data on patients with hypercalcemia, their use in differential diagnosis of such states may be misleading.

METHODS

Intravenous Calcium.—The procedure utilized was that reported by Howard, Hopkins, and Connor.[7] Patients were given a constant, low-calcium diet for 3 days; 24-hour urine samples were collected daily; and on the morning of the second day an infusion of saline containing 12 to 15 mg. of calcium (as calcium gluconate—glucoheptonate) per kilogram of body weight was administered in 4 hours. During this day, and often on the first control day as well, blood was obtained at 4-hour intervals for determination of calcium and phosphorus.

Tubular Reabsorption of Phosphate.—This procedure was carried out after an overnight fast. The patients were hydrated orally and kept in bed during the test. Voided urine was collected for 2 consecutive 1-hour periods (an occasional female patient was catheterized). Blood for determination of calcium, phosphorus, and creatinine was obtained during the period of urine collection. Despite recognized limitations of the method, glomerular filtration rate (GFR) was determined for each period by the clearance of endogenous creatinine.[16] Tubular reabsorption of phosphate (TRP), expressed as per cent of that filtered and as milligrams per 100 ml. glomerular filtrate (GF), was calculated in accordance with previous suggestions.[10, 12, 17, 18] The duplicate collections provided a guide as to the reproducibility of results by this technique. Restriction of dietary phosphorus, a procedure which increases the per cent but apparently not the amount of phosphorus reabsorbed by the renal tubules,[10] was not attempted.

Administration of Cortisone or Related Steroids.—The route of administration and dosage were not constant. When possible, cortisone was given orally at 12-hour intervals according to the following schedule: 300 mg. for one day, then 200 mg. daily for 5 days followed by 100 mg. daily for 5 or more days. In a number of patients similar doses of cortisone or hydrocortisone were given intramuscularly. Prednisone was used in the occasional patient with heart disease.

Chemical Determinations.—Calcium was determined by the Clark and Collip modification of Kramer and Tisdall's method,[19] inorganic phosphorus by the Umbreit[20] modification of the method of Fiske and Subbarrow,[21] and creatinine in serum and urine by the method of Peters.[22]

RESULTS

Intravenous Calcium.—The changes occurring in serum and urine of control patients given calcium intravenously have been described previously[6, 7] and consist essentially of the following: by the fourth hour after completion of the infusion, the concentration of serum phosphorus increases 1.4 to 3.0 mg./100 ml.; the 24-hour urine phosphorus content decreases from 18 to 66 per cent of that obtained on the control day; and the urinary calcium increases on the test day by an amount equal to 35 to 55 per cent of the injected dose.*

*These authors[6, 7] interpreted the changes in serum and urine of such patients as follows: As a rise in concentration of serum phosphorus occurs in hypoparathyroid patients and also in normal individuals given calcium intravenously, this increase would seem to be due largely to a shift of inorganic phosphorus from cellular to extracellular fluid. The decrease in urinary phosphorus occurring in normal patients after calcium administration does not occur in patients with hypo- and hyperparathyroidism, and thus in normals is possibly a reflection of decreased parathyroid hormone secretion consequent to the induced hypercalcemia.

Calcium has been administered intravenously to 18 patients with hyperparathyroidism (proved subsequently by operation) and 10 patients with hypercalcemia due to other causes. (Detailed observations on these and other patients given calcium intravenously are to be reported separately.[23]) In all 28 of these patients the responses were similar and differed from those occurring in normal individuals. The maximal increase in concentration of serum phosphorus was usually less than 1.1 mg. per cent.* The urinary phosphorus on the day of infusion was either only slightly less (0 to 20 per cent) or actually more than on the control day. The changes occurring in hypercalcemic and normocalcemic patients given calcium intravenously are illustrated by the examples in Fig. 1. The observations on patients with hyperparathyroidism

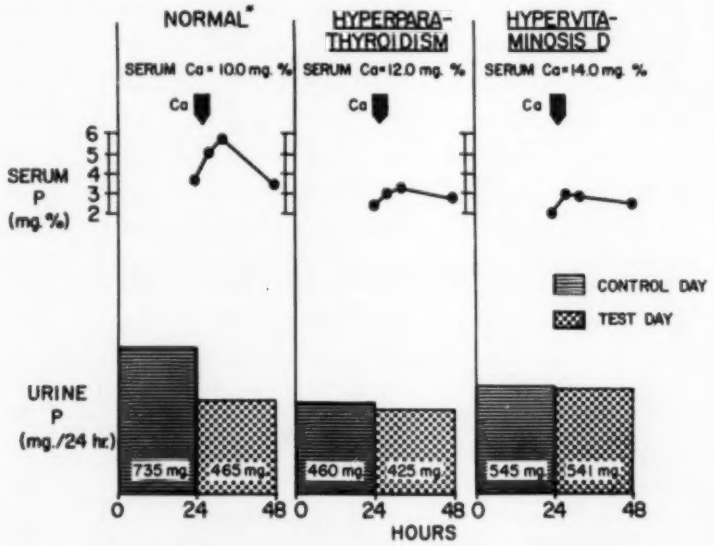

Fig. 1.—Alteration in serum and urine phosphorus after calcium administration. *Data on normal patient previously reported.[3]

are in accord with those previously reported.[6-10] In the 10 patients with hypercalcemia due to causes other than hyperparathyroidism the concentration of serum calcium was 12 mg. per cent or greater. In 4 additional patients with various disorders in whom the concentration of serum calcium ranged from 11.0 to 11.9 mg. per cent the response to calcium intravenously was normal in two and like that in patients with a greater degree of hypercalcemia in the remaining two. That the response to calcium intravenously is nearly identical in patients with hyperparathyroidism and in those with hypercalcemia of other etiology is not surprising and will be discussed subsequently.

*Although in one patient with hyperparathyroidism the phosphorus concentration of the serum increased 1.6 mg. per cent, the urinary phosphorus in this patient increased 11 per cent on the day of infusion rather than decreased as would be expected in normal persons.

*Tubular Reabsorption of Phosphate (TRP)**.—The results obtained in 3 groups of patients are recorded in Table II. The TRP in 7 patients who did not have azotemia or hypercalcemia ranged from 84 to 91 per cent of the

TABLE II. TUBULAR REABSORPTION OF PHOSPHATE IN NORMOCALCEMIC AND HYPERCALCEMIC PATIENTS

NO. OF PATIENTS	PATIENT	DIAGNOSIS	SERUM Ca (MG. %)	SERUM P (MG. %)	NPN (MG. %)	P REABSORBED (MG. /100 ML. GF)	TRP (%)
7		Normocalcemia	<10.7	2.8-4.6	28-32	2.1-4.4	84-91
8	V. B.	Hyperparathyroidism	12.2	2.1	29	1.2	59
	H. B.	Hyperparathyroidism	12.8	2.1	25	1.6	75
	J. C.	Hyperparathyroidism	11.6	2.2	26	1.9	86
	E. G.	Hyperparathyroidism	12.0	2.4	28	1.9	82
	J. W.	Hyperparathyroidism	15.9	2.3	37	1.3	55
	E. W.	Hyperparathyroidism	13.4	2.0	37	1.2	63
	S. P.	Hyperparathyroidism	15.5	1.7	25	0.7	43
	M. O.	Hyperparathyroidism	11.5	1.6	35	1.1	66
7	W. M.	Carcinoma of lung	16.5	3.3	52	1.9	57
	J. T.	Multiple myeloma	17.8	4.3	77	2.5	59
	J. W.	Intraperitoneal carc., primary site?	15.2	2.0	45	1.1	57
	A. C.	Hypervitaminosis D	11.0	3.4	40	2.7	81
	J. C.	Carcinoma of lung	15.5	3.0	29	1.5	52
	F. D.	Multiple myeloma	14.0	3.2	83	1.2	37
	C. H.	Carcinoma of bladder	15.0	3.2	53	1.9	62

filtered phosphorus, values which are in agreement with those reported for normals by this method.[10, 12, 24] In 8 patients with hyperparathyroidism, only two of whom had slight azotemia, the tubular reabsorption of phosphorus varied from 55 to 86 per cent, whereas in 7 patients with hypercalcemia of other etiology the values were also low, ranging from 37 to 81 per cent. In this latter group of patients azotemia was present in all except one, and the creatinine clearance was in general much lower in these patients (17 to 64 ml. per min.) than in those with hyperparathyroidism (61 to 114 ml. per min.). The phosphorus reabsorption expressed as milligrams reabsorbed per 100 ml.

*Urinary phosphorus is currently considered to represent that phosphorus filtered by the glomeruli, but not reabsorbed in the renal tubules.[23, 29] That the tubules may actually secrete phosphorus must be considered a possibility pending more definitive studies. In one patient with hypophosphatemia[27] and several with markedly impaired renal function[28] available data does suggest the possibility of tubular secretion of phosphorus.

glomerular filtrate in the 3 groups of patients was as follows: normocalcemic patients, 2.1 to 4.4 mg.; patients with hyperparathyroidism, 0.7 to 1.9 mg.; and those with hypercalcemia of other etiology, 1.1 to 2.7 mg. Thus, phosphorus reabsorption calculated either as per cent TRP or as mg. per 100 ml. glomerular filtrate was quite similar in the 2 groups of hypercalcemic patients.

The clearance of endogenous creatinine does not approximate glomerular filtration rate in patients with impaired renal function [16, 17] and this may have introduced considerable error in some of the above calculations.* However, the data of Goldman and Bassett[28] demonstrate that a markedly reduced glomerular filtration rate is accompanied by a relative increase in phosphorus excretion, and this may account for the low TRP in those patients with hypercalcemia of nonparathyroid origin.

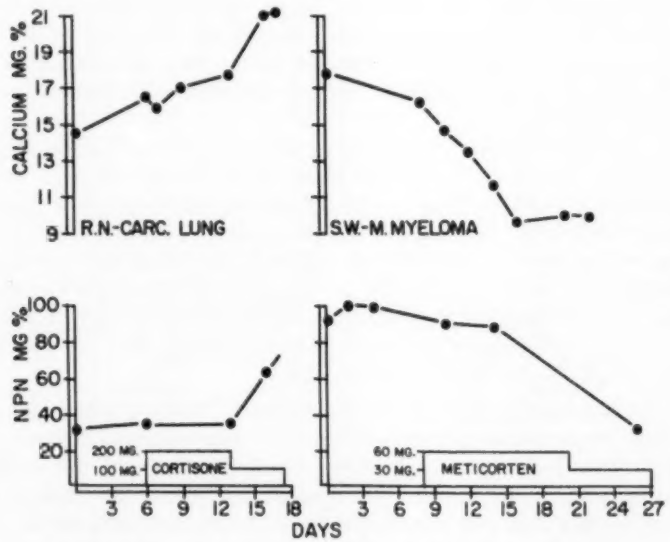

Fig. 2.—Effect of adrenal steroids in patients with hypercalcemia.

Administration of Cortisone or Related Steroids.—During the past 3 years 15 patients with hypercalcemia of known etiology have been given cortisone, hydrocortisone, or prednisone. In 3 patients with hyperparathyroidism and 6 patients with hypercalcemia of other etiology this therapy did not lower the concentration of serum calcium. The 6 remaining patients, none of whom had hyperparathyroidism, became normocalcemic after 5 to 14 days of treatment. Illustrative examples of the inconsistent response on steroid administration to hypercalcemic patients are given in Fig. 2.

*To circumvent such errors one may simply use the phosphorus clearance to express over-all transport of phosphorus through the kidney. By this means, also, the values obtained in both groups of hypercalcemic patients were similar.

DISCUSSION

Present evidence suggests that hormone output by the parathyroid glands is determined by the concentration of ionized calcium in extracellular fluid.*[29] Hypercalcemia apparently reduces the activity of the parathyroid glands,[7, 31, 32] whereas hypocalcemia increases secretion of parathyroid hormone.[33] That such responsiveness does not exist in primary hyperparathyroidism is obvious, for, if so, patients with this disorder would themselves correct the hypercalcemia.[7] Conversely, in hypercalcemia due to other causes the parathyroid glands should be functionally at rest. Thus, with respect to the amount of circulating parathyroid hormone, individuals with hypoparathyroidism may be analogous to those with hypercalcemia of nonparathyroid origin. If these tenets be valid, the essentially identical results obtained on administration of calcium intravenously to patients with hypo- and hyperparathyroidism,[6, 7] and to those with hypercalcemia due to other causes, become explicable in that in each disorder the amount of circulating parathyroid hormone would not be altered by the calcium infusion. Moreover, these observations confirm previous predictions as to the expected results in patients with hypercalcemia of nonparathyroid origin.[6]

That parathyroid hormone affects urinary phosphorus excretion is well known,[34-36] and most,[10-13] but not all[10, 37] (J. D., Table II), patients with primary hyperparathyroidism have a reduced tubular reabsorption of phosphate. The patients with hypercalcemia due to other causes (Table II) also had a low phosphate reabsorption. In these latter patients poor renal function probably accounted for the results obtained.[28] Although hypercalcemia is known to be deleterious to renal function,[4] the data of Schaaf and Kyle[12] and of Hiatt and Thompson[38] would indicate that transient hypercalcemia does not specifically impair the tubular reabsorption of phosphate.

The studies herein reported were conducted in hypercalcemic patients as they were encountered and without regard to existing renal disease. Many of these patients had markedly impaired renal function and the results obtained indicate that caution must be exercised in utilizing the phosphorus reabsorption as an aid in differential diagnosis of hypercalcemic states. Moreover, impaired reabsorption of phosphate has been noted in patients with a variety of disorders in addition to those mentioned. Among these are patients with Cushing's syndrome, cirrhosis of the liver, resistent rickets, myxedema,[13, 39] the Fanconi syndrome,[40] and renal calculi.[37] Thus, it appears that in any disorder in which either phosphorus metabolism or renal function is affected, tubular reabsorption of phosphate may be low.

Cortisone administration has been found effective in reducing the concentration of serum calcium in many, but not all, patients with hypercalcemia of nonparathyroid cause.[15] To our knowledge adrenal steroid administration has not lowered the calcium concentration in any patient with

*Whether or not other factors influence secretion of parathyroid hormone is not yet known. Some authors[29] consider the concentration of serum phosphorus to be one controlling factor in the secretion of parathyroid hormone.

hyperparathyroidism.[14, 15] Such observations incite speculation as to the mode of action of cortisone in these disorders,[41–43] but are of limited value in the differential diagnosis of patients with hyperparathyroidism.

As the 3 procedures discussed have been of little value to us in differentiating patients with hyperparathyroidism from those with other causes of hypercalcemia, other means of establishing the correct diagnosis have been utilized. Reliable laboratory determinations and expert interpretation of roentgenograms have been indispensable. Although hypophosphatemia commonly exists in patients with hyperparathyroidism who are not azotemic, it has also been frequently noted in those with hypercalcemia of other causes, e.g., vitamin D intoxication,[44] the milk-alkali syndrome,[45] multiple myeloma,[46] carcinoma, Hodgkin's disease,[47] and rarely in sarcoidosis.[48] There is a high incidence of peptic ulcer in patients with hyperparathyroidism,[49] but this, too, is not of diagnostic value for ulcerlike symptoms occur frequently in patients with hypercalcemia of any origin.[5, 50]

Recent study of a large series of patients with hyperparathyroidism has furnished several additional criteria of occasional use in establishing the diagnosis.[51] Resorption of bone at the distal end of the clavicles may precede other distinctive radiologic manifestations of hyperparathyroidism.* Furthermore, the CO_2 combining power is normal (less than 28 mEq./L.) or low in patients with hyperparathyroidism, whereas, in those with hypercalcemia of other etiology it is often high. (Of course marked azotemia may be accompanied by a low CO_2 combining power in both groups of patients.) Finally, when the evidence suggests that the disorder has existed for many years, hyperparathyroidism is more likely than other causes of hypercalcemia which are rarely of such duration. Thus, until a reliable and sensitive assay method for parathyroid hormone becomes available, the clinical diagnosis of hyperparathyroidism depends, at least in our experience, on careful evaluation of all relevant data and an awareness of other disorders which may at times mimic this endocrinopathy.

SUMMARY

To establish the diagnosis in any particular hypercalcemic state may be exceedingly difficult. Recently, the response to calcium intravenously, tubular reabsorption of phosphate, and the effect of cortisone administration have been used in the study of hypercalcemic patients, but the results reported herein suggest that these procedures are of limited aid in differential diagnosis of such patients. Certainty in the recognition of hyperparathyroidism prior to surgical exploration is highly desirable, and several observations of possible assistance in this regard are discussed.

We wish to express our appreciation to the many house officers of the Departments of Medicine, Surgery, and Urology, Johns Hopkins Hospital, whose cooperation made these studies possible. In addition we wish to thank Mr. Harry Eisenberg and Miss Sue Fulford for technical assistance.

*We are indebted to Dr. David Gould, Department of Radiology, University of Arkansas Medical College, for these observations.

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BLOOD CLEARANCE AND TISSUE DISTRIBUTION STUDIES IN THE DOG FOLLOWING INTRAVENOUS ADMINISTRATION OF RADIOACTIVE COLLOIDAL GOLD (AURCOLOID)

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WITH increasing clinical use of radioactive colloidal gold, continuing studies of the disposition of this material in species other than man are indicated. Relatively few studies of this type have been published. In addition to possible clinical application, information obtained in such studies may increase our fundamental knowledge of the phagocytic process.

Radioactive colloidal gold is usually used clinically by some form of local administration, such as intracavitary. Because of the following considerations, only the intravenous route was used in our experiments:

1. The intravenous route has been used clinically in attempts to reach internal sites of deposition unobtainable with local forms of administration.

2. Systemic toxic effects have been reported following local forms of administration, indicating that escape into the general circulation may occur.[1]

3. The process of systemic phagocytosis can be most conveniently studied by introducing the particulate material directly into the general circulation.

For a variety of reasons, to be discussed, only the commercially available gold colloid preparation, Aurcoloid, was used in our experiments, although most of the previous investigators in this field prepared their own colloid.

MATERIALS AND METHODS

Randomly selected mongrel dogs of both sexes were anesthetized with sodium pentobarbital, administered intravenously in a dose of 25 to 30 mg. per kilogram and both femoral veins were exposed. At the time of manufacture, the specific activity of the gold colloid solution varied from 20 to 40 mc. per milliliter.[2] This solution was then diluted with isotonic saline to achieve a final concentration of approximately 2 mc. per milliliter. A dose of 2 to 4 mc. in a volume of 1 to 5 ml. was carefully injected into one of the femoral veins, the exact amount depending upon the radioactivity present at the time of injection. Aspiration of blood into the syringe both before and after injection ensured that the radioactive materials were introduced into the general circulation. The injection was made rapidly, in a period of less than 5 seconds. At various time intervals following the injection, blood samples were

From the Division of Pharmacology, University of Tennessee, Medical Units Division, Memphis, Tenn., and the Medical Division, Oak Ridge Institute of Nuclear Studies, Oak Ridge, Tenn. The work at Memphis was performed under Atomic Energy Commission Contract No. AT-(40-1)-1640.

The radioactive colloidal gold (Au^{198}) was prepared by Abbott Laboratories, North Chicago, Ill., and Oak Ridge, Tenn.

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*Research Participant, Medical Division, Oak Ridge Institute of Nuclear Studies, during summer of 1952.

taken from the opposite femoral vein. Volumes of 1 ml. were pipetted from each of the blood samples into 10 ml. volumetric flasks and were brought to volume with water, effecting hemolysis. The entire 10 ml. solution was transferred to a standard container and the radioactivity determined using a Geiger-Muller tube with an end window thickness of 3.5 mg./cm.^{2} to count the 0.97 MEV beta ray emitted by Au^{198}. The animals were sacrificed by exsanguination 1 to 1½ hours following injection. The various organs and tissues to be analyzed were quickly removed. They were blotted to absorb excess blood. Fat and other extraneous tissues were carefully dissected away, and the organs and tissue were weighed. The gall bladder was carefully dissected away from the liver before the latter was weighed. Tissue samples routinely analyzed included liver, kidney, spleen, lung, bone marrow, heart, and skeletal muscle. All tissues were heated with nitric and hydrochloric acid until a homogeneous digest was obtained. This acid digest was quantitatively transferred to a volumetric flask of suitable size and was brought to volume using water and acetone. Dioxane was added to reduce the explosive hazards of acetone additon to the hot acid digests. A suitable aliquot was removed from the volumetric flask, diluted to the standard volume of 10 ml., transferred to a standard container, and counted as described above. All samples were counted under identical conditions of geometry for at least 5,000 counts or 10 minutes, whichever occurred first. Since it was inconvenient, because of its size, to digest the entire liver, two portions weighing approximately 50 grams each were obtained after the entire organ had been weighed, and were analyzed separately. Close agreement of these duplicate liver determinations indicated that this procedure resulted in a representative sample of liver tissue being obtained.

The analytical results have been expressed in a number of ways to facilitate comparisons. Results of the blood clearance studies are expressed as T ½ and T 1/10 values to indicate the time required for the radioactivity values to fall to 50 and 10 per cent, respectively, of the original, extrapolated value presumably existing immediately following injection. Tissue results have been expressed as the percentage of the injected dose per organ for discrete tissues and as the percentage of injected dose per kilogram of tissue for both discrete and nondiscrete tissues. Corrections for background and for radioactive decay were made on all of the counting data. Statistical treatment of the data involved calculation of the standard error.

TABLE I. RATE OF REMOVAL OF RADIOACTIVITY FROM CIRCULATING BLOOD STREAM OF THE DOG FOLLOWING INTRAVENOUS ADMINISTRATION OF AUROCOLOID

DOG NO.	T ½ (MINUTES)	T 1/10 (MINUTES)
1	1.50	4.0
2	2.00	6.5
3	1.75	6.0
4	1.50	4.7
5	2.25	7.5
6	1.50	5.4
7	2.25	7.0
8	1.40	4.75
9	2.00	6.4
10	1.75	5.9
11	2.00	6.5
12	1.8	5.8
Total No. of animals	Mean value for T ½ ± S.E.	Mean value for T 1/10 ± S.E.
12	1.80 ± 0.093	5.86 ± 0.33

RESULTS

Blood Clearance Studies.—In agreement with results reported previously by other investigators,[3, 4] it was found that intravenously administered radioactive colloidal gold disappeared rapidly from the circulating blood stream.

Table I gives the T ½ and T $\frac{1}{10}$ values of the animals studied as well as the standard error of these values. It can be seen that the blood disappearance rate is not only rapid but is also remarkably constant from animal to animal.

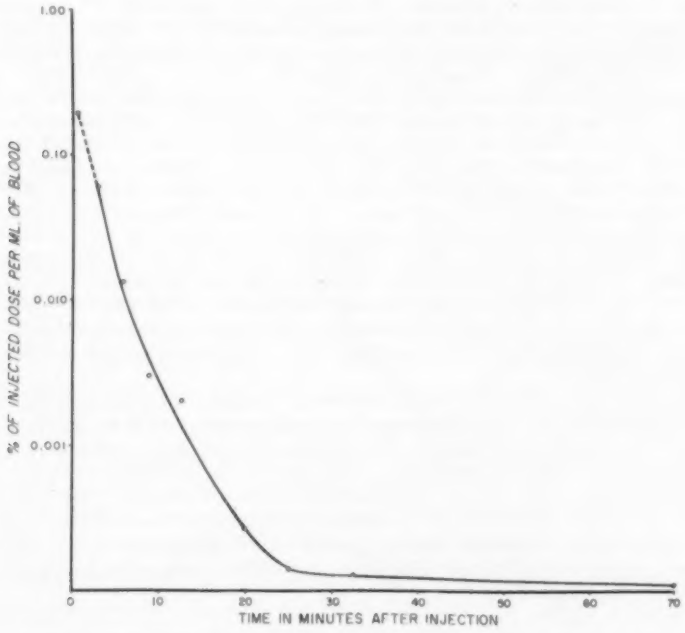

Fig. 1.—Representative blood disappearance curve following intravenous administration of Aurcoloid, extrapolated to "O" time value.

Fig. 1 is a representative, semilogarithmic curve of the blood clearance rate in which the level of radioactivity in the blood stream is plotted against time after injection. The removal process consists essentially of two phases—an initial phase in which the blood radioactivity falls sharply to a very low value, followed by a second phase in which a very low level of blood radioactivity is fairly well maintained for a much longer period of time. Smooth disappearance curves were obtained in all but one of our experiments. In one experiment, however, a sharp rise in the blood radioactivity level was found in the 50-minute blood sample, a sample which would normally be expected to show very little radioactivity. The animal in question was in a condition of severe respiratory depression at this time with tachycardia and an extremely weak pulse pressure. Manual artificial respiration was being administered. A possible explanation for the reappearance of a high blood radioactivity level in this animal is that the shocklike condition was one of extreme stress to the animal and produced a disturbance in reticuloendothelial function manifested by extrusion of

previously phagocytized particles back into the blood stream. Warner and Dobson[5] have recently demonstrated an extrusion phenomenon using colloidal chromic phosphate and severe thermal injury as the stress situation.

Tissue Distribution Studies.—Following intravenous administration, Aurcoloid rapidly leaves the blood stream to be deposited primarily in those organs and tissues having known reticuloendothelial functions. Table II presents results that have been calculated as the per cent of injected dose per organ and clearly indicates the overwhelming importance of the liver in concentrating intravenously administered colloidal gold. The small variability in this respect from animal to animal is shown by the relatively low value for the standard error. The second organ of importance is the spleen which takes up approximately 2 per cent of the injected dose. This value, while low in comparison to the liver, is important in that it is approximately 20 times as great as the third ranking organ, the lungs. The ratio of the standard error to the mean value indicates that there is more variability in spleen uptake than in liver uptake. The lungs and kidneys take up smaller amounts of radioactivity, while the value for the heart is negligible.

TABLE II. TISSUE DISTRIBUTION IN DOGS FOLLOWING INTRAVENOUS ADMINISTRATION OF AURCOLOID, CALCULATED AS PERCENTAGE OF INJECTED DOSE PER ORGAN

ORGAN	NO. OF ANIMALS	AVERAGE VALUE	STANDARD ERROR
Liver	12	94.1	1.21
Spleen	10	1.96	0.193
Lungs	10	0.091	0.0092
Kidney	11	0.027	0.0033
Heart	10	0.0049	0.0008

TABLE III. TISSUE DISTRIBUTION IN DOGS FOLLOWING INTRAVENOUS ADMINISTRATION OF AURCOLOID, CALCULATED AS PERCENTAGE OF INJECTED DOSE PER KILOGRAM

TISSUE	NO. OF ANIMALS	AVERAGE VALUE	STANDARD ERROR
Liver	12	380	8.05
Spleen	10	43.9	5.59
Lung	10	1.00	0.232
Kidney	11	0.94	0.091
Heart	10	0.084	0.021
Skeletal muscle	6	0.023	0.0088
Bone marrow	5	8.38	1.59

Table III presents tissue distribution data calculated on a concentration per kilogram basis rather than on a concentration per organ basis. This type of calculation is valuable for two reasons. First, it makes possible the determination of radioactivity in those tissues which are not discrete organs, such as bone marrow and skeletal muscle. Significant bone marrow uptake can thus be demonstrated. Second, it makes comparisons possible on a pure weight basis, eliminating organ size as a factor. It is interesting to compare the results listed in Table II with those in Table III. For example, from Table II, the liver-spleen ratio on a per organ basis is roughly 50. On a per gram basis, however, the ratio is sharply reduced to a value of approximately 9. This type of comparison shows that the spleen is actually much more efficient in concentrating

colloidal gold than the per cent of injected dose per organ results would indicate and that the low value of 2 per cent in this respect is to a considerable extent the result of its much smaller weight compared to the liver. Thus, in terms of the intrinsic phagocytic ability per gram of tissue, the importance of the spleen is much more clearly seen.

The above considerations apply as well to comparisons between the lungs and kidneys. In Table II the lung value is approximately 3 times the value of kidney. On a per gram basis, however, those two organs are roughly equivalent, indicating that organ size is primarily involved since the average weight of the lungs is about 3 times that of the kidney.

Results of analyses of two additional tissues, skeletal muscle and bone marrow, which are not included in Table II because they are not discrete organs, are found in Table III. Skeletal muscle concentration of Aureoloid is extremely low. Bone marrow, on the other hand, shows relatively good uptake, as might be expected because of the known reticuloendothelial properties of this tissue, with a value of roughly $\frac{1}{5}$ that of the spleen and $\frac{1}{40}$ that of the liver.

DISCUSSION

Comparison of the work reported here with previously reported results of other investigators is difficult because of differences in experimental conditions. Varying the method of preparation can produce gold colloids with different physical properties. Alterations in metabolism have been reported from the use of gold colloids with different particle sizes. Zilversmit and associates[4] have shown, for example, that large particle size gold colloids are removed more quickly from the circulation than colloids containing smaller sized particles. These investigators found no differences, however, in tissue distribution. On the other hand, Mayneord and Sinclair[7] reported that the ratio of concentrations in liver and spleen is strongly dependent upon the size of the colloidal particles. Inasmuch as metabolic alterations apparently can result from the use of colloids with different physical properties, we felt that use of a highly standardized and conveniently available gold colloid preparation, such as Aureoloid, was most desirable.

Other differences in experimental conditions that could contribute to differences in experimental results include the species and number of animals used, analytical procedures employed, the dosage, and the time period following injection after which the animals were sacrificed.

Some similarities and differences between our results and those of other investigators will now be more closely examined. In practically all of the work previously reported the liver is recognized as the site of greatest systemic uptake. A quantitative difference appears, however. The average value of the per cent of injected dose for this organ in the 4 animals reported by Zilversmit was 83 per cent. Our results on 12 animals gave an average of 94 per cent. A recent study in which rats were used reports values of 88.7 and 95.8 per cent; the two figures represent results from studies of two gold colloids of different specific activities.[6] Additional evidence for the higher liver values can be found in the

work of Little and Kelly[8] who studied radioactive colloidal gold uptake by the isolated, perfused liver and reported that 85 per cent alone is taken up by the first passage of blood through this organ.[8]

Although the secondary importance of the spleen is recognized by most investigators, Mayneord and Sinclair state that the spleen normally has the highest concentration of gold. We have never found the spleen to contain a higher concentration of colloidal gold than the liver.

The rate of disappearance from the circulation was practically identical with that reported by Zilversmit for gold particles of approximately 40 mμ in diameter. On the basis of electron microscope data, the particle size of the colloid used in our work is reported to be but 3 mμ.[2] Zilversmit reported a much slower blood clearance rate for particles of this small size. This discrepancy may be resolved by recognizing that the effective particle size of Aurcoloid may be considerably larger than 3 mμ due to the presence of a gelatin coating around the colloidal particle, resulting from the use of gelatin as a colloid stabilizer in the manufacturing process.

When plotted on semilogarithmic paper, the blood disappearance curve in all but one of our experiments was a smooth curve with few or no irregularities, or "bumps." Zilversmit attributed smooth disappearance curves in his experiments to the presence of one or both of the following experimental conditions: (1) use of a gold colloid prepared by using yellow phosphorus and H_2O_2 as reducing agents and (2) premixing the gold colloid with the dogs' own serum proteins prior to injection. Since our experiments were not performed under either of these conditions, we feel that they are not essential to obtaining a smooth disappearance curve.

Because of the known phagocytic potentialities of the circulating white blood cells it was somewhat surprising to find their apparent lack of participation in gold colloid phagocytosis as evidenced by the almost complete disappearance of radioactivity from the circulation in a matter of minutes following injection. A separate study of this situation has been undertaken using in vitro and in vivo techniques and a preliminary report has been published.[9]

SUMMARY

1. A study of the fate of intravenously administered radioactive colloidal gold (Aurcoloid) in the dog is presented with statistical evaluation of the results.

2. The liver is of predominant importance with respect to systemic uptake, accumulating approximately 94 per cent of the injected dose.

3. Only liver, spleen, and bone marrow appear to have the ability to concentrate significant amounts of the injected colloid.

4. The injected material disappears very rapidly from the circulating blood stream in an essentially smooth, two-phase process.

5. The effective particle size of the gold colloid used (Aurcoloid) appears to be about 40 mμ.

6. Comparisons of our results with those of other investigators are discussed.

We acknowledge with gratitude the technical assistance rendered by Mr. Wallace Luke and Mrs. Penny Jennings.

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TRYPTOPHAN METABOLISM IN NORMAL AND DIABETIC SUBJECTS

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TRYPTOPHAN has assumed increasing importance in recent years with the recognition of abnormalities in its intermediary metabolism in a wide variety of diseases, including diabetes,[1] tuberculosis,[2] certain anemias,[3-5] cancer,[6] malignant carcinoid,[7] pyridoxine deficiency,[8-10] and phenylketonuria.[11]

Following oral administration of tryptophan to diabetic subjects, the urinary excretion of xanthurenic acid was found to be increased[1]; this abnormality was also observed in diabetic rats,[12] accompanied by a decrease in urinary N^1-methylnicotinamide.[13] Furthermore, tryptophan has been implicated in the regulation of blood sugar as the precursor of an insulinase inhibitor.[14]

In order to obtain a fuller picture of the fate and role of this amino acid in diabetes, the effect of an oral load of tryptophan on the urinary excretion of tryptophan, kynurenine, anthranilic acid, xanthurenic acid, and 5-hydroxyindoleacetic acid has been observed in normal and diabetic subjects. For this purpose an 8-hour procedure was established which, it is thought, may be of value in the assessment of tryptophan metabolism for clinical purposes in other conditions, as suggested by Bessey.[15]

MATERIALS AND METHODS

Three groups of subjects were studied: (1) 4 "young" men and 7 "young" women aged 25 to 35, normal volunteers from the laboratory staff, (2) 6 older women aged 50 to 75, in whom no organic disease was demonstrable, and (3) 12 older women aged 50 to 75 with uncomplicated diabetes. For each subject the dietary intake of thiamine, riboflavin, and niacin, as estimated by the short dietary history method,[16] met the requirements of the Canadian Standards of Nutrition.[17] Pyridoxine intake could not be calculated because of the lack of reliable data on the pyridoxine content of foods. The young women were studied in the preovulatory phase of the menstrual cycle to minimize any effects of variations in titer of reproductive hormones.

Experiments were performed in the morning after an overnight fast; the protocol consisted of the collection of a 2-hour (basal) urine specimen, the oral administration of 4 Gm. of L-tryptophan, and collection of urine for the subsequent 6-hour period. Preliminary studies had revealed that the excretion rates of the various metabolites obtained in the basal period were maintained during the subsequent 6-hour period and that 70 to 90 per cent of the tryptophan recovered in the form of the metabolites under study was excreted within 6 hours of tryptophan administration. Consequently, the amount of each metabolite excreted

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in response to the test dose was taken as the difference between the quantity excreted following tryptophan and that predicted for a 6-hour interval from the 2-hour basal period. In the two groups of older subjects, blood specimens were taken for glucose estimation at 1-hour intervals and for tryptophan and kynurenine at 2-hour intervals after administration of the tryptophan.

The method of Ichihara and associates[18] was adapted for analysis of urine. This gave 3 fractions: tryptophan plus unknown diazotizable substances (UDS) anthranilic acid (including conjugates), and kynurenine. Tryptophan was estimated separately by application of the above procedure to the precipitate obtained by the addition of mercuric sulfate to urine.[19] The value so obtained was subtracted from that of the first fraction noted above; the remainder of this fraction consisted of UDS and was expressed in terms of tryptophan equivalent. Recoveries from urine of tryptophan were in the range of 93 to 104 per cent, of anthranilic acid 93 to 99 per cent, and of kynurenine 92 to 99 per cent.

The method developed by Glazer and associates[20] was used for the estimation of urinary xanthurenic acid. Although the color intensity did not obey the Beer-Lambert law, the recoveries calculated from a standard curve were 95 to 108 per cent for amounts of xanthurenic acid ranging from 17 to 308 μg in a 1 ml. aliquot of urine. Since values obtained from urine under basal conditions were largely due to nonspecific chromogens, they have not been given in the results. The increases in values following administration of tryptophan, however, were considered to be entirely due to xanthurenic acid. 5-Hydroxyindoleacetic acid (5-HIAA) in urine was measured by a modification of the procedure described by Macfarlane and co-workers.[21] Recoveries of 5-HIAA added to urine were in the range of 99 to 105 per cent for quantities of 15 to 135 μg 5-HIAA in a 10 ml. aliquot.

Blood glucose was estimated by the Somogyi modification of the Nelson method.[22] Slight modifications of the Ichihara method were applied to the measurement of tryptophan and kynurenine in plasma.

TABLE I. THE EXCRETION OF TRYPTOPHAN AND ITS METABOLITES IN THE 6-HOUR PERIOD FOLLOWING THE ADMINISTRATION OF 4 GM. L-TRYPTOPHAN (YOUNG NORMAL SUBJECTS)

	NO.	SEX	TRYPTOPHAN (MG.)	KYNURENINE (MG.)	ANTHRANILIC ACID (MG.)	XANTHURENIC ACID (MG.)	UDS*
Basal (2 hours)	4	M	2.6 ± 0.3†	1.2 ± 1.0†	2.1 ± 0.5†		6.9 ± 2.3†
	7	F	2.6 ± 0.3	1.1 ± 0.1	1.1 ± 0.1		9.4 ± 1.7
Increment	4	M	20.9 ± 2.0	34.7 ± 14.6	3.9 ± 1.5	19.2 ± 4.1	67.8 ± 19.8
	7	F	85.6 ± 23.3	114.5 ± 22.8	28.2 ± 11.1	25.2 ± 11.1	109.8 ± 50.2
Percentage of administered tryptophan recovered in 6 hours	4	M	0.5 ± 0.0	0.8 ± 0.3	0.1 ± 0.1	0.5 ± 0.1	1.7 ± 0.5
	7	F	2.1 ± 0.6	2.8 ± 0.5	1.1 ± 0.4	0.6 ± 0.3	2.7 ± 1.4

The increment in excretion of tryptophan and its metabolites attributed to the ingested tryptophan is calculated as: (excretion in 6 hours following tryptophan) − (3 × basal excretion).

*Unknown diazotizable substance calculated as tryptophan.

†Mean Excretion ± S.E.M.

RESULTS

The results obtained in young normal individuals are shown in Table I. It is seen that there was no sex difference in the basal excretion for any of the substances measured, but after the administration of tryptophan the increase in excretion of all metabolites except xanthurenic acid was greater in the women.

The values obtained in the older diabetic and nondiabetic subjects are presented in Tables II and III. Three hours following ingestion of the tryptophan, there was a statistically significant lowering of blood sugar in the diabetic subjects ($p < 0.05$) but it was of minor degree. There was no difference in serum tryptophan levels between the groups, but the kynurenin levels were distinctly lower in diabetic patients than in normal individuals ($p < 0.01$).

TABLE II. CHANGES IN BLOOD SUGAR, TRYPTOPHAN, AND KYNURENINE AFTER INGESTION OF 4 GM. L-TRYPTOPHAN IN 6 CONTROL AND 12 DIABETIC SUBJECTS

		TIME AFTER INGESTION (HOURS)					
	SUBJECTS	FASTING	1	2	3	4	6
Sugar (capillary blood) (per cent of fasting)	Control	100	104 ± 2	103 ± 1	102 ± 2		
	Diabetic	100	102 ± 2	97 ± 3	92 ± 4		
Tryptophan (serum) mg. /100 ml.	Control	1.2 ± 0.1		10.1 ± 0.7		6.2 ± 0.4	3.2 ± 0.4
	Diabetic	1.2 ± 0.1		9.1 ± 0.8		5.8 ± 0.8	3.4 ± 0.4
Kynurenine (serum) mg./100 ml.	Control	0.05 ± 0.05		0.82 ± 0.22		0.94 ± 0.18	0.42 ± 0.08
	Diabetic	0.04 ± 0.02		0.25 ± 0.08		0.19 ± 0.06	0.23 ± 0.07

Values shown are M ± S.E.M.

TABLE III. URINARY EXCRETION OF TRYPTOPHAN AND ITS METABOLITES BEFORE AND AFTER INGESTION OF 4 GM. L-TRYPTOPHAN IN 6 CONTROL AND 12 DIABETIC SUBJECTS

	BASAL (2 HOURS) (MG.)	INCREMENT* (MG.)	PERCENTAGE DOSE ACCOUNTED FOR
Tryptophan			
Control	3.0 ± 0.6†	74.9 ± 17.3†	1.9 ± 0.4†
Diabetic	2.9 ± 0.5	33.0 ± 3.8	0.8 ± 0.1
Kynurenine			
Control	2.3 ± 0.7	203.9 ± 61.8	5.0 ± 1.5
Diabetic	1.5 ± 0.4	43.8 ± 9.5	1.1 ± 0.2
Anthranilic acid			
Control	1.4 ± 0.3	34.2 ± 10.4	1.3 ± 0.4
Diabetic	1.1 ± 0.2	12.9 ± 2.6	0.5 ± 0.1
Xanthurenic acid			
Control		58.8 ± 20.3	1.5 ± 0.5
Diabetic		15.3 ± 3.4	0.4 ± 0.1
5-Hydroxyindole-acetic acid			
Control	0.29 ± 0.05	1.27 ± 0.21	0.03 ± 0.01
Diabetic	0.29 ± 0.05	1.53 ± 0.20	0.04 ± 0.01
UDS‡			
Control	7.0 ± 2.3	90.7 ± 41.8	2.3 ± 1.0
Diabetic	8.7 ± 1.8	78.4 ± 19.9	1.8 ± 0.5

*Increment = (Excretion in 6-hour interval after ingestion of tryptophan) − (3 × basal excretion).
†M ± S.E.M.
‡Unknown diazotizable substances.

The basal urinary excretion of each metabolite measured was the same in the two groups. However, the diabetic patients excreted much less of the administered dose of tryptophan in the form of tryptophan ($p < 0.01$), kynurenine ($p < 0.01$), anthranilic acid ($p < 0.05$), and xanthurenic acid ($p < 0.01$). They continued to excrete the same amounts of 5-HIAA and of UDS as the control subjects.

There was no consistent difference between the results in the "young" and "old" women.

DISCUSSION

One of the purposes of this study was to establish a method of assessing tryptophan metabolism which might be of clinical use. Bessey has stressed the value, in the diagnosis of pyridoxine deficiency, of a simple procedure which consisted of the administration of a tryptophan load and subsequent measurement of urinary xanthurenic acid.[14] The procedure described in the present report allows a more complete assessment of tryptophan degradation and should therefore have a wider range of applicability. For example, we have observed 2 subjects with suboptimal intakes of riboflavin who showed increased excretions of kynurenine and anthranilic acid, suggesting the presence of a block in the conversion of kynurenine to hydroxykynurenine; since this chemical transformation is known to be dependent on riboflavin,[23] it is suggested that the present tryptophan loading test may offer a simple means of detecting subclinical deficiency of this vitamin.

The chemical methods employed are straightforward and reproducible; they give results in good agreement with those obtained by more elaborate and refined procedures,[24] but do not distinguish between free and conjugated anthranilic acids.

In the group of nondiabetic subjects the differences between men and women are probably attributable, at least in part, to differences in lean body mass, since the average body weight of the men was considerably greater than that of the women.

The differences between diabetic and nondiabetic subjects, however, are of a more fundamental nature. Although in any oral loading test the factor of rate of absorption from the gut cannot be adequately evaluated, it is unlikely that this contributed to the differences in the present case since the blood tryptophan levels following the load in the diabetic subjects were almost identical with those of the control group, implying a normal rate of absorption.

McDaniel and associates[13] reported a decrease in the excretion of N^1-methylnicotinamide in alloxan diabetes, and suggested that this and the increased excretion of xanthurenic acid were due to an impaired ability to remove the sidechain of kynurenine and 3-hydroxykynurenine. It might be anticipated that such a block would lead to a decrease in anthranilic acid excretion and an increase in kynurenine excretion (Fig. 1). In the present work there was indeed a decrease in anthranilic acid excretion, but there was also a decrease in kynurenine excretion of even greater degree. The latter change, and the decrease in serum kynurenine levels would not be compatible with the concept of a metabolic block at the kynureninase level.

Furthermore, a decrease occurred in xanthurenic acid excretion rather than an increase as had been reported earlier.[1] Results similar to the present ones were obtained by Joron in a group of 100 patients (unpublished data).

These discrepant results emphasize the observation of Hawkins and coworkers[25] regarding the great variability of xanthurenic acid excretion after tryptophan loading.

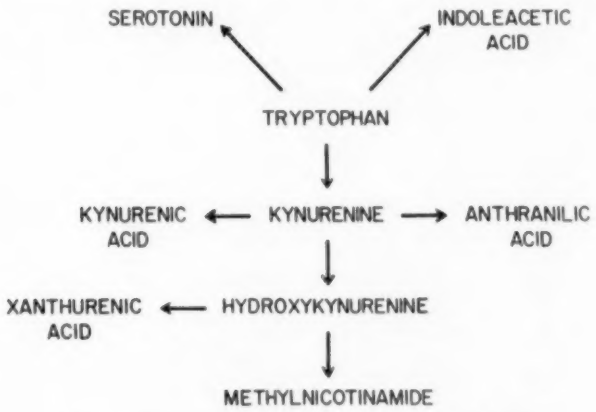

Fig. 1.—Metabolic pathways of tryptophan metabolism.

There are two ready explanations of the abnormalities observed in the diabetic subjects: (1) there may have occurred a diversion of tryptophan from the nicotinamide pathway, resulting in a lowered production and excretion of all metabolites along this route; (2) the rate of degradation starting along the nicotinamide pathway may have been normal or increased, but diverted, as postulated by Dalgliesh and associates,[26] to complete oxidation. It is not possible to decide between these alternatives on the basis of evidence presently available.

Mirsky and associates[14] have reported that the oral administration of tryptophan produced a drop in blood sugar concentration in normal rats and a transient rise in sugar concentration in diabetic rats; the hypoglycemic effect was attributed to an anti-insulinase action. McDaniel[13] confirmed the effect in diabetic rats but observed no change in normal animals; the transient hyperglycemia was thought to be due to gluconeogenesis from the tryptophan. In the present work tryptophan did not alter the blood sugar in control subjects, but produced a slight decrease in diabetic patients. The differences between these results and those obtained in animal studies may be related to differences in relative dose. However, since 4 Gm. of tryptophan had only a slight effect on the blood sugar concentration it is difficult to conceive of any physiologically important role for tryptophan in regulating glycemia when the usual daily intake is only 0.5 to 1.0 Gm.

SUMMARY

A procedure has been described which permits an evaluation of tryptophan metabolism suitable for clinical purposes; it is based on a measurement

of the excretion of various tryptophan metabolites following ingestion of tryptophan. Diabetic and nondiabetic subjects have been studied by this means to determine the effect of diabetes on tryptophan metabolism and the effect of tryptophan on glycemia. Diabetic subjects were found to excrete a subnormal proportion of the administered load in the form of tryptophan, kynurenine, anthranilic acid, and xanthurenic acid, but normal amounts of 5-hydroxyindoleacetic acid. Tryptophan effected a minor decrease in blood sugar concentrations in diabetic subjects but not in normal individuals.

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THE PRECISION OF PERCENTILES IN ESTABLISHING NORMAL LIMITS IN MEDICINE

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PERHAPS the most difficult problems in the establishment of normal limits or ranges in medicine are the problems concerning definition and the selection of subjects. However, while we do not wish to underestimate the seriousness of these problems, the scope of the present paper is limited to a discussion of methods applied to the already collected data.

Purposes of Normal Limits.—Norms appear to serve two fairly distinct functions—one is descriptive, the other discriminative. While the anthropometrist may be primarily interested in the descriptive aspect, the clinician is interested in normal limits to the extent that they aid him in diagnosing an individual correctly as either healthy or diseased. Inspection of a single normal range very rarely leads immediately to a correct diagnosis; rather, the normal range acts as a more or less useful screening device, ruling out certain possibilities with more or less high probability. The range will be the more useful the smaller the overlap between the distributions of values in the healthy and in the diseased. Thus, in order to evaluate the usefulness of specific normal ranges in the healthy for diagnostic purposes, it is necessary to examine the ranges of the same variates in disease. Relatively little quantitative work has been done on this question; therefore the clinician must fall back on normal values in the healthy and let his clinical experience be his guide as to their usefulness for diagnosis.

Some Current Methods of Determining Limits of Normal.—The most common method of obtaining normal limits in clinical medicine at the present time appears to be that of taking the mean $\pm$ 2 standard deviations.[1] Another common way of setting normal limits is by means of the lowest and highest values found in a sample of measurements. The mean $\pm$ 2 standard deviations purports to give a "95 per cent normal range" in which 2.5 per cent of the normal subjects have values below the mean minus 2 standard deviations and another 2.5 per cent have values above the mean plus 2 standard deviations. This is true (1) if the measurements form the mathematical distribution which is called the Gaussian and often, misleadingly, the "Normal" distribution, and (2) if the mean and standard deviation are estimated from very large samples, so

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that they may be considered known quantities. In general, therefore, the mean and standard deviation cannot be considered known. (Naturally, it is possible to make allowance for sampling variation of the mean and standard deviation, i.e., if the sample is large enough to satisfy one that the Gaussian distribution is a fair approximation.) Moreover, with relatively small samples, we simply cannot tell whether or not the parent population may be considered Gaussian; while it may sometimes be close to the Gaussian distribution, the assumption that biologic measurements generally have this distribution is both unwarranted and dangerous. Using the mean ± 2 standard deviations on a distribution which is skew or, if symmetrical, either peaked or flat, can lead to a serious misclassification of cases, depending on the extent of deviation from the Gaussian shape.

The defect of the method of using the lowest and highest sample values as the limits of normal is simply that it underestimates the 95 per cent range in small samples, while it overestimates it in large samples. If sample sizes are given, it is possible to determine the extent of under- or overestimation for a particular range.

The Percentile Method of Determining Normal Limits.—The drawback of the "extreme range" method (that is, taking the highest and lowest values) is overcome by the percentile method, in which, regardless of sample size, there is no persistent under- or overestimate of, say, the 95 per cent range. By this method in order to obtain an estimate of the 95 per cent range such that 2.5 per cent of the observations in the population lie below the lower limit and another 2.5 per cent lie above the upper limit, one simply finds the estimates of the population 2.5 and 97.5 percentiles in the form of the corresponding sample percentiles. The sample 2.5 percentile can be most simply described as the value that cuts off the lowest 2.5 per cent of the observed measurements; other percentiles are analogously defined (for more detail, see below).

The remainder of this paper will be devoted to showing how to determine the reliability of certain sample percentiles and thus enable the investigator to know in advance what sample size is required for a specified amount of precision. The beauty of the method is that *no assumptions are required about the distribution shape* (Gaussian or other) of the population from which the sample is drawn. The method should particularly appeal to clinical investigators who have become convinced of the importance of local norms, which must often be based on small samples. Local norms consist of standards set up in a particular location and may be restricted to persons with closely specified characteristics. (It may be said that every norm is a local norm to a greater or lesser degree.)

The method described here for the use of clinical investigators is not new. It was first proposed by Thompson,[6] and subsequently more exhaustively studied by Wilks[3, 4] who also prepared some short tables for use in industrial quality control work. In 1948, Murphy[5] published graphs which have a very general application but which are not in a form that the medical investigator would find useful nor, indeed, do they include certain applications very important in clinical work.

For the present, we decided against the use of the 2.5 and 97.5 percentiles. The reason is that, for screening procedures in which the potentially ill person is to be studied more intensively, it seems preferable to draw the range of acceptance (as healthy) somewhat narrower, as is done with bodily dimensions.[2] Thus, there would be a greater chance of detecting abnormal subjects (the diseased) in situations where there is a good deal of overlap between the distribution of values in the healthy and diseased. We have, therefore, substituted the estimation of the tenth and ninetieth percentiles. Two other limits of normal are also considered in detail.

The cases which are designated by a chosen limit as being in the tail areas of a particular population distribution, that is, outside the range chosen as normal, will be referred to as the *excluded* cases.

Method of Estimating Percentiles and Determining Their Precision.—To illustrate the use of Tables II and III, Dr. Elaine P. Ralli of the Department of Medicine, New York University College of Medicine, has kindly given us permission to use some serum total protein determinations made in her laboratory. The determinations were made on 49 volunteer medical students between the ages of 19 and 31, by the micro-Kjeldahl method. The values obtained are shown in Table I.

TABLE I. SERUM TOTAL PROTEINS
(Gm. per 100 ml. of serum, micro-Kjeldahl)

6.0	7.2	7.5	7.7	8.0
6.5	7.2	7.6	7.7	**8.0**
6.9	7.2	7.6	7.8	**8.1**
7.0	7.2	7.6	7.8	**8.2**
7.0	7.2	7.6	7.8	**8.3**
7.0	7.3	7.6	7.8	8.4
7.1	7.3	7.6	7.8	8.4
7.1	7.4	7.6	7.8	8.5
7.1	7.5	7.6	7.9	8.5
7.1	7.5	7.7	7.9	

Boldface figures are the estimated upper and lower tenth percentiles.

Estimation of tenth percentile: 1. Order the items from lowest to highest value, letting the lowest value be item No. 1, i.e., the item of rank 1.

2. To find the ordered item estimating the sample tenth percentile, multiply (N + 1) by 10 per cent, where N is the sample size. N + 1 rather than N is used[4] because it can be shown that N + 1 gives the better (unbiased) estimate of a percentile. With N = 49, we see that 10 per cent of 50 = 5.0. Thus the fifth ordered item, the value 7.0 Gm., is the estimate of the tenth percentile. If, for some other sample size, item No. 5.3 had been obtained as the estimate of the percentile, linear interpolation would have been used between the values of the fifth and sixth items. Such interpolation introduces some inaccuracy into the estimation of the percentile and should be avoided if possible by using the sample sizes given in Table II.*

*At the present time, better methods of interpolation are being investigated in this Department by M. I. Sutcliffe.

Estimation of the ninetieth percentile: 1. Treat the ninetieth percentile as the tenth percentile with the items ordered from highest to lowest value, letting the highest value be item No. 1.

2. To determine the value estimating the ninetieth percentile, find the value of the fifth item in the order from highest to lowest value. It is 8.3 Gm.

TABLE II. LIMITS OF POPULATION PERCENTAGE EXCLUDED BY USE OF THE SAMPLE TENTH PERCENTILE*
(Probability level for each limit = 0.975)

SAMPLE SIZE	LIMITS OF PERCENTAGE EXCLUDED	
	LOWER	UPPER
19	1.30	26.00
29	2.19	22.78
39	2.87	20.89
49	3.39	19.61
59	3.83	18.69
69	4.17	17.97
79	4.47	17.42
89	4.73	16.94
99	4.95	16.56
109	5.15	16.22
119	5.31	15.93
129	5.47	15.67
139	5.62	15.46
149	5.75	15.27
159	5.86	15.07
169	5.98	14.91
179	6.07	14.76
189	6.16	14.62
199	6.25	14.50
209	6.29	14.38

*For explanation of terms and method of use of table, see the accompanying examples.

Precision of the estimate of the tenth percentile: 1. Enter Table II with sample size N = 49.

2. Opposite 49 find the percentages 3.39 and 19.61. The percentage 3.39 is the lower limit of the population percentage excluded by the sample tenth percentile, which means that there is a low probability (only 1 in 40 or P = 0.025) that less than 3.39 per cent of the population values are below the sample tenth percentile. Similarly, the upper limit, 19.61, signifies that there is a probability of 1 in 40 that more than 19.61 per cent of the population values are below the sample tenth percentile. Putting these two expressions together, we see that there is a probability of 1 in 20 (P = 0.05) that a sample tenth percentile will exclude less than 3.39 per cent or more than 19.61 per cent of the population values. Exactly the same statement can be made about the sample ninetieth percentile, if we remember that the population percentage excluded refers to the population values which are *higher* than the value of the ninetieth percentile.

Table II shows the wide variability for the population percentage excluded by the sample tenth percentile in small samples which are, therefore, often almost useless for estimates of this kind. However, it may well be possible to

obtain clinically useful information from small samples by use of Table III which shows the sample percentiles required to ensure with high probability the exclusion of (1) more than 5 per cent of the population values and (2) less than 15 per cent of the population values. The exclusion of at least 5 per cent of the standard (healthy) population may be desirable in screening for differential diagnosis when certain symptoms are actually present. Although a relatively large proportion of the healthy will be in the "excluded" group (and therefore studied more intensively as being potentially ill) when the method is applied to a small sample, the likelihood of a healthy person presenting himself for clinical diagnosis may be sufficiently small to warrant the use of this

TABLE III. SAMPLE PERCENTILE REQUIRED TO ENSURE THE EXCLUSION OF SPECIFIED POPULATION PERCENTAGES*
(Probability level = 0.975)

	POPULATION PERCENTAGE TO BE EXCLUDED			
	MORE THAN 5 PER CENT		LESS THAN 15 PER CENT	
SAMPLE SIZE	REQUIRED SAMPLE PERCENTILE	REQUIRED RANK	REQUIRED SAMPLE PERCENTILE	REQUIRED RANK
20	17.77	3.73	–†	–†
23	16.75	4.02	4.26	1.02
30	15.09	4.68	5.13	1.59
40	13.53	5.55	6.04	2.48
50	12.49	6.37	6.75	3.44
60	11.75	7.17	7.32	4.47
70	11.18	7.94	7.78	5.52
80	10.72	8.68	8.17	6.62
90	10.35	9.42	8.50	7.74
100	10.04	10.14	8.78	8.87
110	9.78	10.86	9.04	10.03
120	9.55	11.56	9.27	11.22
130	9.35	12.25	9.46	12.39
140	9.17	12.93	9.64	13.59
150	9.01	13.61	9.80	14.80
160	8.86	14.26	9.96	16.04
170	8.73	14.93	10.09	17.25
180	8.62	15.60	10.21	18.48
190	8.51	16.25	10.33	19.73
200	8.41	16.90	10.43	20.96

*For explanation of terms and method of use of table, see the accompanying examples.
†For sample sizes under 23 there is no sample percentile which would ensure, with P = 0.975, an exclusion percentage of less than 15 per cent.

standard. The exclusion of at least 5 per cent of the standard population may also be desirable, when it is of interest to study those subjects of a small sample who are likely *not* to be in the extreme parts of the population distribution, i.e., those who make up the bulk or average of the population. On the other hand, the exclusion of no more than 15 per cent of the healthy population may be useful in the screening of groups consisting primarily of healthy individuals among whom the proportion to be followed should be kept low. Naturally, if there is considerable overlap between the healthy and diseased populations, only the more extreme deviations in the diseased population will be identified correctly.

In order to determine what a required sample percentile corresponds to, in terms of an actual measurement, we must rank the measurements from lowest to highest and express the percentile as a rank. To save the reader the trouble of making the conversion, percentiles as well as ranks are given in Table III (with two decimal places for satisfactory interpolation).

The exclusion of at least 5 per cent of the population values: 1. For N = 49 enter Table III with N = 40 and N = 50.

2. Opposite N = 40 and N = 50 find the required sample percentiles (13.53 and 12.49, respectively) as well as the ranks (5.55 and 6.37) of the values corresponding to those sample percentiles. The sample percentiles are the ones which ensure, for their respective sample sizes, that the population percentage excluded by them will be at least 5 per cent, i.e., there is a probability of only 1 in 40 that such sample percentile will exclude less than 5 per cent of the population values.

3. Interpolate linearly between the two ranks to obtain the rank of the value required for N = 49, namely, 5.55 + 9/10 (6.37 − 5.55) = 5.55 + 0.74 = 6.29. Round to the first decimal place. (If the percentile form is desired, we have here the 12.58 percentile: [r] [100]/[N + 1] = [6.29] [100]/50 = 12.58.)

4. To find the value of item No. 6.3, interpolate linearly between the values of the sixth and seventh items, 7.0 Gm. and 7.1 Gm. Since grams of total protein were determined to the nearest first decimal only, it is not necessary to carry the linear interpolation beyond the first decimal; therefore, the value of item No. 6.3 can be taken as 7.0 Gm. For the upper end of the distribution, the sixth and seventh items from the top are 8.2 Gm. and 8.1 Gm., respectively, and item No. 6.3 is taken as 8.2 Gm.

The exclusion of no more than 15 per cent of the population values: 1. For N = 49 enter Table III with N = 40 and N = 50.

2. Opposite N = 40 and N = 50 find the required sample percentiles (6.04 and 6.75) as well as the ranks (2.48 and 3.44) of the values of those percentiles. The sample percentiles are the ones which ensure, for their respective sample sizes, that the population percentage excluded by them will be no more than 15 per cent, i.e., the probability that such sample percentile will exclude more than 15 per cent of the population values is only 1 in 40.

3. Interpolate linearly between the two ranks to obtain the rank of the value required for N = 49, namely, 2.48 + 9/10 (3.44 − 2.48) = 2.48 + 0.86 = 3.34. Round to the first decimal place. (Item No. 3.34 is the 6.68 percentile.)

4. To find the value of item No. 3.3, interpolate linearly between the values of the third and fourth items, 6.9 and 7.0 Gm. Since values to the first decimal place only are required, item No. 3.3 may be taken as 6.9 Gm. For the upper end of the distribution, the third and fourth items from the top are both 8.4 Gm., and therefore the value of item No. 3.3 would be taken as 8.4 Gm.

Treatment of Values Falling Exactly on the Estimated Limits.—After we have determined the normal limit which meets our requirements, the one remaining question is whether individuals who have values equal to that limit should be included among the normal or should be considered "suspect." A general mathematical discussion of this kind of problem by Scheffé and Tukey[7] substantiates the following approach to our particular situation.

1. We have the statement that the sample value required to ensure, with P = 0.975, the exclusion of less than 15 per cent of the population in the lower end of the distribution is 6.9 Gm. of total serum protein. If we draw the normal limit immediately *below* 6.9 Gm., it follows that we shall have even more assurance (higher than P = 0.975) of excluding *less* than 15 per cent of the population. The question of how high the probability may be[8] is more complicated and of less practical importance and, therefore, will not be discussed here.

2. We have the statement that the sample value required to ensure, with P = 0.975, the exclusion of more than 5 per cent of the population in the lower end of the distribution is 7.0 Gm. of total protein. Now, if we draw the normal limit immediately *above* 7.0 Gm., we shall have an even higher probability (higher than P = 0.975) of excluding *more* than 5 per cent of the population. Again, the question of how high the probability may be will not be discussed here.

The application of the above rules to limits at the upper end of the distribution will be obvious. Moreover, the rules cope with the problem that arises when, in the sample used for estimating the limits, two or more determinations have the value of the limit itself; for instance, in the case of the total proteins the value 7.0 Gm. appeared 3 times and the value 8.4 Gm. appeared twice.

Experimental Verifiability of Limits for the Excluded Population Percentage.—It is possible to verify the limits of variability of the excluded population percentages experimentally. Visualize many different standard populations from each of which a random sample of 49 is taken to determine a particular limit of normal. (Alternatively, one may visualize repeated random samples of 49 taken from the same standard population.) Then, to take an example, we obtained from Table III the information that item No. 3.3 is required to ensure, with P = 0.975, that less than 15 per cent of the population values will be excluded. Experimentally this means that if the value of item No. 3.3 is determined for every one of the random samples and we had an opportunity to check back into the populations from which they came, we would find, on about 97.5 per cent of the occasions, that the items would exclude less than 15 per cent of the population values. In addition, we could also verify the statement that item No. 3.3 (the 6.6 sample percentile, determined by the use of N + 1) is an unbiased estimate of the population 6.6 percentile, because we would find that the average or mean population percentage excluded by all the observed sample percentiles was about 6.6 per cent.

Note on Mathematical Derivations.—It may be of interest to some to consider briefly how it is possible to find mathematically the limits of the population percentage excluded by use of a given sample percentile or, conversely, to find the sample percentile such that the limit of the population percentage excluded is a certain desired value. Visualize a continuous population arranged from lowest to highest value in which the lowest p_1 of the values is called the A's, the remainder the non-A's. Further visualize the taking of a random sample from this population. This random sample may again be ordered from lowest to highest value and may contain some items from the lowest p_1 of the population values, i.e., some A's, and some non-A's. Then the probability that a certain item in the sample, e.g., the rth lowest item, excludes a population proportion *less* than (or equal to) the lowest p_1 of that population is equal to the probability that r or more of the sample items are A's, when the population contains a proportion of p_1 A's. But this probability is well known; it is simply the sum of certain binomial probabilities, i.e., $\sum_{A=r}^{N} \binom{N}{A} p_1^{A} q_1^{N-A}$, where Σ stands for summation, N for the sample size, and $q_1 = 1 - p_1$. This expression can be used in 2 ways; we can fix r, say, at the tenth sample percentile, and then find the p_1 for which the sum of these probabilities adds up to the desired 0.025 value (or some other value) or we can fix p_1 at some desired value, say 0.05 and solve for r, corresponding to some sample percentile, such that the sum of these binomial probabilities is again 0.025.

Similarly, the probability that the rth lowest item in a random sample excludes a population proportion *greater* than p_2 is the same as the probability of drawing a sample containing $r - 1$ or fewer A's from a population containing a proportion of p_2 A's. (The lowest p_2 of the population is now called the A's.) Again, this is simply the sum of the binomial probabilities $\sum_{A=0}^{r-1} \binom{N}{A} p_2^{A} q_2^{N-A}$; if r is fixed, then we can find the p_2 for which the sum of these probabilities adds up to 0.025, and if p_2 is fixed, we can solve for r.

Computational Note.—The basic source for the entries in Tables II and III is 95 per cent binomial confidence limits as published by Mainland, Herrera, and Sutcliffe.[9] For Table II, sample sizes were so chosen that $(0.10)(N + 1)$ was a whole number, which meant that only direct interpolation for N was needed. Table III required inverse interpolation for N, which usually resulted in fractional values. The tabulated integer values for N were obtained by linear interpolation between the logarithms of N and the logarithms of the required exclusion percentage.

The entries in Tables II and III may be considered good in the first decimal place. However, since the second decimal place often does contain some information, it has been tabulated and may be used in interpolations.

Other Normal Limits.—The reader may wish to estimate the reliability of normal limits not given in this paper, e.g., the fifth percentile or the twentieth percentile. These can also be obtained by the use of binomial confidence limit tables.[9*]

SUMMARY

The percentile method of determining normal limits is discussed. The primary advantage of this method is that the reliability of sample percentiles can be determined without making any assumptions about the distribution shape (Gaussian or other) of the population from which the sample is drawn.

Tables are given for (1) assessing the reliability of the tenth sample percentile in terms of limits of excluded population percentages, and (2) determining the sample percentiles required to ensure the exclusion of specified population percentages (more than 5 per cent, less than 15 per cent). Sample sizes covered in the tables range between 20 and 200.

*Copies of instruction sheets used in this laboratory will be supplied on request.

Some clinical laboratory data are used to illustrate in detail the method of estimating percentiles and the use of the tables.

This study arose from the work on norms by Dr. Donald Mainland whom I wish to thank for his encouragement and constructive criticism. I also am indebted to both Dr. Donald Mainland and Miss Marion I. Sutcliffe for a very careful reading of the manuscript.

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THE EFFECTS OF AMINO ACID INGESTION ON GASTRIC SECRETION: COMPARISON OF LYSINE AND ARGININE

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THE original objective of this series of studies was the elucidation of certain favorable effects on nutrition which had been reported by previous workers to result from the addition of L-lysine monohydrochloride to the diet.[1-3] Part of the program was the determination of specific physiologic effects of lysine, if any could be determined. In previous papers,[6, 7] we reported that L-lysine monohydrochloride had a gastric secretagogue effect, particularly relating to an increased secretion of pepsin. Certain questions remain unanswered. Are the effects observed specific for lysine? Are they in some way related to the chemical nature of this substance rather than to its biologic physiology? Might not the same effects be produced by other amino acids? Because arginine, like lysine, is a basic amino acid and available as a monohydrochloride salt, it was selected for employment in a second series of human experiments intended to answer some of these questions.

MATERIAL AND METHODS

The subjects were young adults, 12 women and 7 men in the arginine series, and 14 women and 7 men in the lysine series. Two series of observations were made on each subject. The first consisted of fractional gastric analysis after a test meal consisting of homogenized and toasted white bread in water. After an interval of about one week, a second series of tests followed the administration of an identical test meal to which 5 Gm. of either L-lysine monohydrochloride or L-arginine monohydrochloride had been added. Samples of gastric contents were analyzed prior to the administration of each test meal and 15, 30, and 60 minutes after its administration. All analytical procedures followed the techniques described by Hawk, Oser, and Somerson.[8]

The significance of the results of analyses after the 2 test meals was tested using the following formulas:

$$t = M_2 - M_1 \sqrt{\frac{N_1 N_2 (N_1 + N_2 - 2)}{(N_1 + N_2) \Sigma X^2}}$$

$$t = M - M \sqrt{\frac{n (N - 1)}{\Sigma X^2}}$$

$$t = \frac{M_1 - M_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$$

t was calculated for 99 per cent probability from Fischer's table.
M = Sum of the samples divided by total number of samples.
N = Number of samples.

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X = Individual sample.
X – M = Deviation of the individual from the mean.
P = Values charted from Fischer's table.
ΣX^2 = Sum of the squares.
Standard deviation, 6, equals square root of the sum of the squares, divided by N.
S.E. equals S.D. divided by square root of N.

RESULTS

The results of the test meal with and without added arginine are given in Table I. Statistical analysis of the amount of hydrochloric acid and pepsin secreted after the 2 test meals indicates that the differences observed have no significance; i.e., the calculated t value does not exceed the value of t in the 0.01 chart for the figure N – 2. It thus can be concluded that, in the volunteers tested, the addition of arginine monohydrochloride to a test meal did not produce either a diminution or an increase in hydrochloric acid or pepsin secretion.

TABLE I. SUMMARY OF LABORATORY OBSERVATIONS (ARGININE SERIES)

CASE NO.	HYDROCHLORIC ACID*								PEPSIN†							
	TEST MEAL				TEST MEAL PLUS ARGININE				TEST MEAL				TEST MEAL PLUS ARGININE			
1	0	5	29	42	33	0	5	44	100	58	339	77	400	64	85	144
2	18	0	2	25	18	0	16	23	50	0	170	56	134	0	169	134
3	45	17	27	59	32	0	4	42	110	110	96	131	230	70	125	230
4	27	13	32	48	29	6	30	45	155	26	48	51	250	0	99	154
5	16	12	34	0	5	4	38	20	85	155	144	0	125	27	22	85
6	14	4	30	30	10	4	30	40	72	52	352	70	186	6	93	186
7	0	0	0	5	0	0	0	4	0	8	42	58	101	154	37	93
8	19	19	19	59	19	28	50	50	30	42	37	109	117	10	134	310
9	7	11	34	61	16	0	14	39	93	37	51	144	125	0	13	58
10	0	0	21	20	0	0	9	13	0	27	197	208	0	37	46	93
11	0	0	0	27	3	0	0	27	93	0	0	85	70	0	37	93
12	28	0	0	58	21	0	22	55	325	51	70	85	144	4	37	125
13	0	0	24	34	0	24	54	83	16	58	64	197	16	197	125	144
14	11	0	24	30	0	36	20	23	109	0	93	125	64	77	85	85
15	0	0	0	11	0	0	19	40	134	6.3	37	101	51	37	85	93
16	0	0	6	2	0	0	0	8	0	0	37	27	4.9	0	25	60
17	21	3	27	15	16	0	3	8	197	41	64	64	144	20	1	16
18	24	3	18	24	0	11	40	53	135	4	58	64	64	4	41	230
19	0	0	42	63	3	0	54	65	6.4	101	154	197	37	64	64	174

*Cubic centimeters of free acid per 100 c.c. secretion at time 0, 15, 30, and 60 minutes.
†Peptic power (Mett) at time 0, 15, 30, and 60 minutes.

The laboratory observations made on subjects given test meals with and without added lysine are listed in Table II. Using the same statistical standards as with the arginine group, the difference between the 2 test meals, as regards both acidity and peptic activity of the gastric secretion induced by them, is found significant.

In Fig. 1 the alterations in hydrochloric acid secretion in 15, 30, and 60 minutes are expressed in relation to the level of the fasting contents. The difference in the height of secretion between the test meal itself and the test meals with arginine and with lysine is represented. Statistical testing of the difference depicted for arginine shows it to be without significance.

In Fig. 2 the effect on peptic secretion of adding arginine or lysine to a standard test meal is depicted. The elevation or depression, compared with

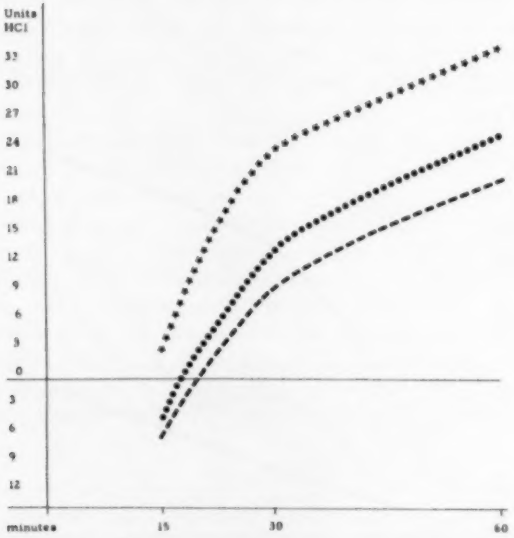

Fig. 1.—Alteration of gastric HCl after test meals with and without added amino acids. Key: — — —, HCl gain or loss over fasting level with test meal; ● ● ●, HCl gain or loss over fasting level with test meal plus arginine; ★ ★ ★, HCl gain or loss over fasting level with test meal plus lysine.

TABLE II. SUMMARY OF LABORATORY OBSERVATIONS (LYSINE SERIES)

CASE NO.	HYDROCHLORIC ACID*								PEPSIN†							
	TEST MEAL				TEST MEAL PLUS LYSINE				TEST MEAL				TEST MEAL PLUS LYSINE			
1	0	0	0	0	0	0	0	0	0	4	13	31	0	46	64	64
2	0	0	0	25	0	0	40	65	27	35	77	230	219	184	282	309
3	6	2	4	9	13	3	30	61	208	19	31	196	243	154	117	243
4	0	0	0	0	0	0	0	0	0	0	0	0	35	0	0	0
5	4	5	12	12	17	0	0	27	125	51	70	91	144	40	16	206
6	0	2	9	22	0	4	16	22	3	58	160	93	77	19	134	195
7	8	24	63	30	8	30	95	105	467	269	336	400	432	99	144	256
8	0	15	64	55	0	37	117	123	174	48	195	296	206	115	269	308
9	0	8	15	20	0	0	20	40	19	27	36	118	42	30	219	309
10	0	3	7	12	20	17	49	46	0	77	118	125	184	115	184	195
11	3	7	19	23	0	0	0	23	144	31	71	135	0	3	19	160
12	0	0	22	8	0	0	21	28	169	36	224	144	64	288	271	361
13	0	15	29	4	0	0	16	30	100	484	400	324	141	144	400	400
14	0	0	23	25	0	0	25	33	81	144	289	210	0	256	576	361
15	0	0	0	0	0	0	16	21	271	0	16	25	185	36	400	400
16	12	15	20	30	0	0	23	36	440	325	197	197	400	64	144	121
17	0	0	12	16	0	0	14	19	324	100	324	400	0	133	324	361
18	0	0	0	0	0	0	8	17	0	4	16	36	25	72	324	460
19	0	0	0	15	0	0	18	30	197	0	101	197	0	560	480	540
20	0	0	0	15	0	8	23	13	0	0	0	144	30	30	64	90
21	0	0	15	0	0	15	30	28	0	36	100	25	25	170	170	677

*Cubic centimeters of free acid per 100 c.c. secretion at time 0, 15, 30, and 60 minutes.
†Peptic power (Mett) at time 0, 15, 30, and 60 minutes.

the fasting level, has been charted at 15, 30, and 60 minutes. It is apparent that a test meal with added lysine produced a greatly increased peptic activity which appeared to be still rising at 60 minutes.

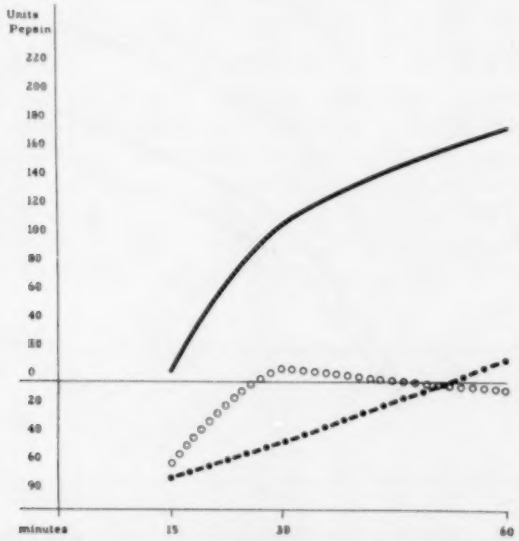

Fig. 2.—Alteration of gastric pepsin after test meals with and without added amino acids. Key: ○○○, pepsin gain or loss over fasting level with test meal; ●—●, pepsin gain or loss over fasting level with test meal plus arginine; ———, pepsin gain or loss over fasting level with test meal plus lysine.

TABLE III. MEAN DIFFERENCES BETWEEN TEST MEALS WITH AND WITHOUT AMINO ACID ADDITION

	ARGININE		LYSINE	
MINUTES	HCl (UNITS)	S.E.*	HCl (UNITS)	S.E.
15	+ 2.8	0.6	−1.1	1.12
30	+ 4.0	0.6	10.8	5.95
60	+ 4.9	0.8	20.0	2.07
	PEPSIN (UNITS)		PEPSIN (UNITS)	
15	−17.6	2.2	53	2.31
30	−56.1	1.7	101	5.35
60	+14.4	1.8	139	8.51

*S.E. $= \sqrt{\frac{(S.D.)^2}{N_1} + \frac{(S.D._2)^2}{N_2}}$

Interpretation of the data presented may be aided by another statistical approach, using the method of paired variates. Calculation of the mean differences in the hydrochloric acid secretion and pepsin secretion, after test meals with and without addition of an amino acid, yields the results that appear in Table III. These results support the conclusion that, used in equal amounts, lysine does and arginine does not stimulate gastric secretion of both hydrochloric acid and pepsin.

DISCUSSION

It is apparent there is a considerable difference between the effect on gastric secretion of L-lysine monohydrochloride and that of L-arginine monohydrochloride. These differences cannot be ascribed to variations in solubility, dissociation, or physical characteristics, and are, therefore, ascribed by us to the specific physiologic roles of the 2 amino acids.[6, 7]

The few observations here reported lend some credence to the hypothesis proposed by some physiologists[4] that the breakdown products of ingested food act in some measure to control the secretion of digestive juices. Thus, Ivy and Javois[5] demonstrated that even a very small concentration of free amino acids in the intestine produce gastric secretion. But it is even more interesting that the present studies indicate that the individual amino acids are dissimilar in action. Further studies might, for example, result in observations indicating that not only do certain amino acids have a gastrosecretagogue effect and others a neutral effect, as with lysine and arginine, but that still others may inhibit gastric secretion. Even with our present incomplete data it is possible to state that the amino acid composition of protein foods in all likelihood have a significant influence upon the degree of gastric secretion evoked by their ingestion.

CONCLUSIONS

1. Arginine monohydrochloride, administered in a dose of 5 Gm. with a bread and water test meal, produced no significant changes in the amount of hydrochloric acid or pepsin detectable.

2. This is in distinction to the effect of lysine monohydrochloride which, given in an identical dose, produced a substantial rise in pepsin and a moderate rise in hydrochloric acid as measured fractionally.

We wish to express our gratitude for the technical assistance of William Garfield, Leonard Garner, and Fredric Heilman.

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OBSERVATIONS ON THE DIAGNOSIS OF TUBERCULOUS MENINGITIS

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BETWEEN August, 1947, and December, 1955, 280 cases of tuberculous meningitis were admitted to the Northern Ireland Fever Hospital. In a review of these cases, certain facts relevant to the diagnosis of the disease have become evident and are offered for consideration.

It is accepted that tuberculous meningitis is essentially progressive, and, if left untreated, the patient pursues a rapidly downhill course. It is of interest to observe how this has been borne out by reference to the cases of this series. A comparison has been made between the severity of the disease on admission to the hospital, and the number of days which had elapsed since the patient first became ill. The assessment of severity was made clinically on the lines of the classification used by the Medical Research Council[1] in their streptomycin trials (1948), each case being designated A (mild), B (moderate) or C (severe) accordingly. The numbers of cases of each severity and the duration of illness are given in Table I. It is apparent that

Table I. Comparing the Severity and the Duration of Illness on Admission

Duration of Illness in Days	Severity of Illness			Total
	A	B	C	
0- 7	24 (72.7)	62 (42.5)	18 (17.8)	104 (37.1)
8-14	7 (21.2)	81 (55.5)	61 (60.4)	149 (53.2)
15-21	2 (6.1)	3 (2.0)	22 (21.8)	27 (9.7)
Total	33 (100)	146 (100)	101 (100)	280 (100)
X^2	35.8146			
Degrees of Freedom	2			
Probability	$P < 0.001$			

In all tables, the numbers in parentheses indicate percentages. It has also been necessary to group certain data to facilitate the X^2 test.

the majority of mild cases were those who were diagnosed within the first week of illness, and that those diagnosed later were, for the most part, in the moderate or advanced stages of the disease. A X^2 test has been applied to the same figures: the result indicates that, at the conventional level of $P=0.05$ (accepted throughout this paper), the differences shown in Table I are greater than are likely to have occurred by chance. It would emphasize, therefore, that there is no such thing in tuberculous meningitis as self-limitation, and it is imperative, especially now that treatment is available, that the diagnosis should be made at the earliest possible moment.

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Diagnosis was based on clinical, laboratory, and other findings. The latter included: cerebrospinal fluid (C.S.F.) sugar and protein estimation, C.S.F. chloride estimation (rarely), C.S.F. cell count, examination of the C.S.F., gastric contents, and urine for tubercle bacilli, the use of the Mantoux reaction, and radiologic examination for evidence of tuberculosis elsewhere in the body (regarded as being presumptive value as to the etiology when the clinical signs and other tests indicated meningeal involvement). It is proposed to deal only with the following data: (1) C.S.F. sugar estimation; (2) C.S.F. protein estimation; (3) examinations for tubercle bacilli; and (4) the Mantoux reaction.

TABLE II. COMPARING SEVERITY OF ILLNESS AND INITIAL C.S.F. SUGAR LEVELS

C.S.F. SUGAR LEVELS (MG. PER CENT)	SEVERITY OF ILLNESS			TOTAL
	A	B	C	
0- 9	3 (9.1)	19 (13.0)	16 (15.8)	38 (13.6)
10-19	3 (9.1)	38 (26.0)	35 (34.7)	76 (27.2)
20-29	5 (15.2)	60 (41.1)	37 (36.6)	102 (36.4)
30-39	8 (24.2)	27 (18.5)	9 (8.9)	44 (15.7)
40 and over	14 (42.4)	2 (1.4)	4 (4.0)	20 (7.1)
Total	33 (100)	146 (100)	101 (100)	280 (100)
Mean	38.7576	25.9452	16.4356	
Standard Error	2.1137	1.0049	1.2082	
X^2	44.4522			
Degrees of Freedom	4			
Probability	$P < 0.001$			

See footnote to Table I.

C.S.F. Sugar Estimation.—It has long been recognized that in tuberculous and other forms of meningitis, the C.S.F. sugar level shows a marked fall, and this has been attributed by Hendry[2] to glycolysis due to the presence of polymorphonuclear leukocytes (the cellular reaction in the C.S.F. in tuberculous meningitis is predominantly lymphocytic, but some polymorphonuclear cells are present especially in the early stages). Somner[3] has claimed that this decrease in sugar "is the most useful single factor in the early diagnosis of tuberculous meningitis," and that "it falls with progressive disease, and rises as the disease process undergoes resolution." Search has been made in the literature for confirmation of this contention. It would appear that this is simply a clinical impression, widely accepted, but without mathematical basis, and it was decided, therefore, to apply the figures pertaining to the 280 cases under review to determine whether they would be supportive or otherwise. The figures obtained at the time of admission to the hospital, or at the time when diagnosis was made, were available for each case, and these have been compared with the severity of the illness as assessed on clinical examination at that time. Was the patient's clinical condition accurately reflected in the C.S.F. sugar level? The results are given in Table II and lend support to this view. The mean sugar value was higher among the mild (severity A) cases and showed a consistent decrease in the more advanced cases (severity B and C). These mean values of C.S.F.

sugar levels and their distributions have been tested by an analysis of variance and X^2 test, respectively, and the apparent differences proved to be of significance.

It is felt that comment should be made on the value of concomitant blood sugar and C.S.F. sugar estimations. The ratio of C.S.F. sugar to blood sugar content should not fall below 0.6:1 under normal circumstances. In tuberculous meningitis, both the C.S.F. sugar level and the ratio of C.S.F. to blood sugar are reduced. Although this fact was pointed out by Hendry[4] several years ago, it does not appear to have been given its proper place as an aid in the diagnosis of the difficult case. In this series, it has been found of most value in those cases with meningeal signs of moderate severity, with a lymphocytic cell reaction in the C.S.F. and without bacteriologic proof as to the causative agent. The lower sugar level and ratio were taken as strong presumptive evidence that the meningitis was, in fact, tuberculous in origin rather than, say, one of the benign lymphocytic forms.

C.S.F. Protein Estimation.—In common with other diseases in which there is a meningeal reaction, the C.S.F. protein level rises in tuberculous meningitis. The question was also studied as to whether or not this rise reflected the severity of the illness. The figures for 257 cases were available and these have been tabulated against the patient's condition as adjudged clinically at the time when the initial lumbar punctures were done. That the protein level rises consistently as severity increases may be seen from comparison of the mean values for severity A, B, and C. That these differences (in mean values and in distribution) are significant has been shown again by an analysis of variance and X^2 test (Table III).

TABLE III. COMPARING SEVERITY OF ILLNESS AND INITIAL C.S.F. PROTEIN LEVELS

C.S.F. PROTEIN LEVELS (MG. PER CENT)	SEVERITY OF ILLNESS			TOTAL
	A	B	C	
0- 99	23 (69.7)	30 (20.7)	20 (25.3)	73 (28.4)
100-199	8 (24.3)	70 (48.3)	25 (31.6)	103 (40.1)
200-299	1 (3.0)	33 (22.7)	16 (20.3)	50 (19.4)
300 and over	1 (3.0)	12 (8.3)	18 (22.8)	31 (12.1)
Total	33 (100)	145 (100)	79 (100)	257 (100)
Mean	75.6364	167.0687	193.4051	
Standard Error	22.8218	10.8866	14.7490	
X^2	39.1336			
Degrees of Freedom	4			
Probability	$P < 0.001$			

See footnote to Table I.

TABLE IV. CORRELATION COEFFICIENT BETWEEN C.S.F. PROTEIN AND SUGAR ESTIMATIONS

Correlation coefficient r	— 0.2222
Value of t	— 3.6289
Degrees of freedom	256
Probability	$P < 0.001$

So far, it has been shown that, in tuberculous meningitis, the C.S.F. sugar content falls with progress of the disease while the C.S.F. protein content tends to rise. Are these two factors accurately correlated? Values of 257

protein estimations, and also for the corresponding sugar estimations (made in the same specimen of C.S.F.), were available. The results of the calculation involved are given in Table IV. This correlation is significant. The negative value of r confirms that the protein and sugar reactions are in opposite directions, i.e., as the protein level rises, the sugar level tends to fall (both in relation to increase in severity of the illness). That the correlation coefficient is small can only be explained by assuming that one or other of the factors involved must be inconstant in its performance as regards severity. This is more likely to be true of protein than of sugar. In the first instance, it is recognized that protein levels are subject to considerable fluctuations such as are not reflected by the sugar levels, e.g., after the administration of intrathecal streptomycin.[5] Another reason is that protein levels are measured in figures reaching as high as several hundreds compared with much smaller figures in the case of sugar levels. It follows that a small technical inaccuracy in making the protein estimation will lead to a greater error in subsequent calculations than if a similar inaccuracy occurred in making the sugar estimation. Furthermore, tuberculous C.S.F. may show a tendency to fibrin clot formation if any time elapses before it is examined; this would also tend to affect the accuracy of the protein estimation without necessarily altering the sugar content. On the whole it may be said that the C.S.F. protein content offers some indication of the severity of tuberculous meningitis, but that this index is less constant and reliable than the C.S.F. sugar content.

Examination for Tubercle Bacilli.—1. In each case in this series, examination of the C.S.F. for tubercle bacilli was made by direct microscopy (Ziehl-Neelson's method) and by culture methods (Löwenstein-Jensen's medium). For initial diagnosis, the latter method is too slow to be of value, but its virtue lies in the fact that certain cases, treated on their clinical merits as tuberculous meningitis, may in the long run be proved, in spite of a perhaps fruitless search for mycobacteria in the stained films. Owing to difficulty in tracing past records, the results of film and culture examinations for tubercle bacilli are available for only 232 and 197 patients, respectively, out of a total of 280. Table V indicates the measure of success in isolating the organism. It is interesting to compare these figures with those published by Harvey[6] in 1950. He reported positive results in 74 per cent of his cases on examination of the film and 76 per cent on culture.

TABLE V. RESULTS OF C.S.F. EXAMINATION FOR TUBERCLE BACILLI

METHOD OF EXAMINATION	NO. OF CASES WITH RESULTS AVAILABLE	NO. OF CASES WITH POSITIVE RESULTS
Direct microscopy	232 (100)	85 (36.7)
Culture	197 (100)	156 (79.2)

See footnote to Table I.

2. Examination of gastric residue: a fasting sample of gastric residue was obtained and cultured for tubercle bacilli.

3. Examination of urine: a sample collected with sterile precautions was also cultured for tubercle bacilli. The results of these two tests are given in Table VI.

TABLE VI. RESULTS OF EXAMINATION OF GASTRIC CONTENTS AND URINE FOR TUBERCLE BACILLI

METHOD OF EXAMINATION	NO. OF CASES WITH RESULTS AVAILABLE	NO. OF CASES WITH POSITIVE RESULTS
Gastric contents culture	145 (100)	31 (21.4)
Urine culture	146 (100)	10 (6.8)

See footnote to Table I.

4. The Mantoux reaction: using Old Tuberculin (0.1 ml. of a 1:1,000 or 1:100 solution) by intradermal injection. It has been pointed out that decreased resistance to infection is sometimes encountered in patients with a severe or advanced form of tuberculosis. It might, therefore, be expected that in cases of tuberculous meningitis, a considerable number of patients would give negative results. Such was not observed; there were only 4 doubtful negative reactions out of a total of 188 patients whose results have been recorded, all the others giving well-defined positive results.

SUMMARY

1. The results are given of some of the investigations conducted on a series of 280 cases of tuberculous meningitis, admitted to the hospital over a period of 8½ years.

2. By presentation of relevant data, emphasis has been laid on the progressive nature of the disease, and on the necessity for early diagnosis.

3. It has been confirmed that the C.S.F. sugar level falls significantly with increase in the severity of the disease, and, conversely, that the C.S.F. protein level rises. Also, that the sugar level is a more reliable guide to severity. These facts, hitherto clinical observations, have been substantiated by submission of the available figures to statistical analysis.

4. The value of estimating the blood sugar level concomitantly with the C.S.F. sugar level as an aid in diagnosis has been stressed.

5. Results are given of examinations of C.S.F., gastric residue, and urine for tubercle bacilli, and mention is made of the results of the Mantoux reaction.

I am indebted to Dr. F. F. Kane, Medical Superintendent, Northern Ireland Fever Hospital, for kind permission to use his cases and to Dr. G. F. W. Tinsdale for access to laboratory records.

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GROUP-SPECIFICITY OF PSITTACOSIS-LYMPHOGRANULOMA VENEREUM GROUP SKIN TEST ANTIGENS IN LYMPHOGRANULOMA VENEREUM PATIENTS

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SKIN test and complement-fixing antigens prepared from lymphogranuloma venereum virus grown in the yolk sacs of embryonated hens' eggs are highly efficient for diagnosis of lymphogranuloma venereum in patients.[1, 2] Treatment of suspensions of lymphogranuloma venereum virus with phenol enhances[3] the complement-fixation titer of the antigen without increase in skin test antigen activity.[4] Such complement-fixing antigen is broadly group-specific in tests with sera from patients with lymphogranuloma venereum, psittacosis, or ornithosis.[3, 5] The present report describes the results of a comprehensive study to measure the group- or species-specificity of phenol-enhanced antigens prepared from 6 viruses of the psittacosis-lymphogranuloma venereum family and tested in human lymphogranuloma venereum patients.

MATERIALS AND METHODS

Antigens.—The psittacosis (6BC), ornithosis (P4), meningopneumonitis (MP-F97), lymphogranuloma venereum (JH), feline pneumonitis (Baker), and mouse pneumonitis (Nigg) viruses employed in the present study have been described elsewhere.[5] The antigens for complement fixation (C.F.) and skin tests were prepared from suspensions of yolk sacs of infected embryonated hens' eggs which were treated with 0.5 per cent phenol at 37° C. for periods up to 8 weeks, to effect maximal enhancement of the C.F. titer.[3, 5] The infectivity of the virus was destroyed by the process and all lots of antigen were checked for safety by passage in 6-day-old embryonated eggs inoculated via the yolk sac.[4] Noninfected yolk sac antigens for control purpose were prepared in identical fashion.

C.F. Test Procedure.—This technique has already been described.[6] The antigens were titrated in half-log steps (i.e., 1:200, 283, 400, 566, 800) to 1:6,400. The antigen titer was the highest initial dilution of yolk sac in the test which gave 75 per cent or more fixation of complement in the test and 1 C.F. unit was contained in 0.25 ml. of that dilution.

Skin Test Titration.—The skin test titrations of L.V. and heterologous antigens were carried out in serologically-proved L.V. patients who reported to the Clinic for treatment. For assay, 0.1 ml. volumes of L.V. antigen containing 0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 C.F. antigen units were injected intradermally on the volar surface of one arm of an L.V. patient and the same numbers of units of a heterologous antigen were injected intradermally in the other arm. Normal control antigen in greater concentration of yolk sac than that of the viral antigens was also given. The range of dilution of infected yolk sac which contained

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from 0.1 to 3.2 C.F. units of viral antigen in 0.1 ml. was 1:283 to 1:18,100. The tests were observed at 2 and 7 days postinjection. A positive test was one in which the axes of the papule formed was 6 mm. by 6 mm. or greater and the result was recorded as the product of the dimensions of the axes in millimeters. The skin test titer was expressed as the least amount of C.F. antigen, in units, which elicited a positive reaction. Each L.V. patient was tested only once in the study.

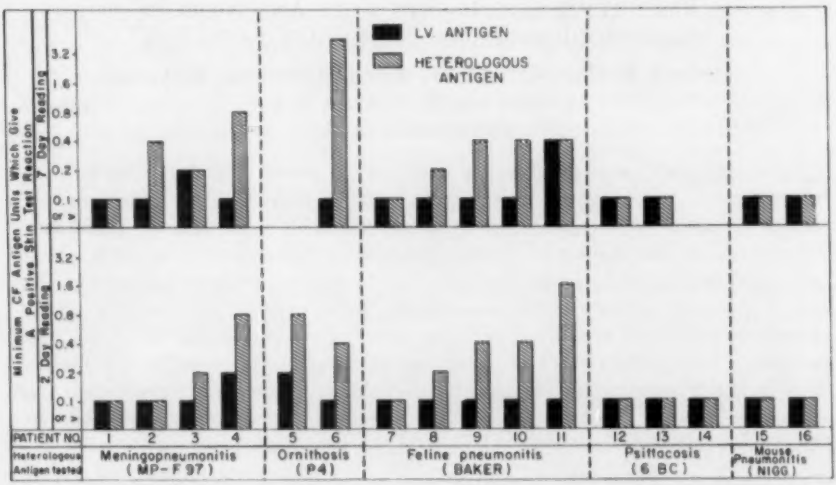

Fig. 1.—Results of skin test titrations of L.V. and heterologous psittacosis-lymphogranuloma venereum group antigens in patients with lymphogranuloma venereum.

RESULTS

The results of the skin test titrations of L.V. antigen and of a heterologous antigen in each of 16 patients are presented in Fig. 1. The black bar designates the least amount of L.V. antigen which elicited a positive skin test reaction in the patient. The cross-hatched bar designates the least amount of a heterologous antigen which gave a positive test in the same patient. It is seen in the figure that the amount of antigen required to elicit a positive skin reaction was usually considerably less than 1 C.F. antigen unit. Further, the various psittacosis-lymphogranuloma venereum group antigens tested were remarkably group-specific in that all 6 viral antigens induced a positive reaction in the L. V. patients. The amount of L.V. antigen needed to give a positive test was extremely small, usually 0.1 C.F. unit or less. The heterologous psittacosis and mouse pneumonitis antigens proved as active as the L.V. antigen in patients 12 through 16 in whom the tests were performed. The heterologous meningopneumonitis, ornithosis, and feline pneumonitis antigens, in most instances, proved less reactive in the skin tests than the homologous L.V. antigen. None of the patients reacted significantly to the uninfected yolk sac control antigen.

Table I and Fig. 2 illustrate further the marked group-specificity of the skin test reactivity of these antigens. A single L.V. patient was injected into the skin of the lumbodorsal surface with 0.5 to 2.0 C.F. units of homologous or heterologous antigen. The amounts of antigen employed were adjusted to

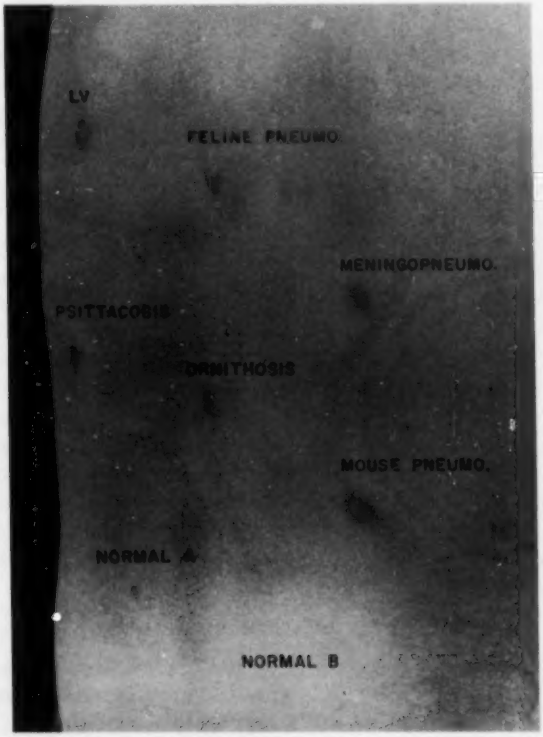

Fig. 2.

TABLE I. SKIN TEST REACTIONS IN A SINGLE L.V. PATIENT TESTED AT ONE TIME WITH 6 PSITTACOSIS-L.V. GROUP ANTIGENS

ANTIGEN		RESULTS EXPRESSED AS PRODUCT OF AXES OF PAPULE IN MM.	
VIRUS	C.F. UNITS EMPLOYED	2-DAY READING	7-DAY READING
L.V.	0.2	81	64
Feline pneumonitis	0.8	72	49
Meningopneumonitis	0.8	56	48
Psittacosis	0.4	49	42
Ornithosis	0.8	63	49
Mouse pneumonitis	0.2	63	63
Noninfected control*			
A	-	0	0
B	-	0	0

*Two different noninfected yolk sac control lots were used.

about twice as much as the estimated minimum required to give a positive result in the skin test titrations. The papules elicited by the different viral antigens were essentially identical in form and were roughly equal in size, the product of the axes ranging from 42 to 81 mm. There was no significant reaction to normal control antigen.

DISCUSSION

Strain-specific differentiation of infection in man with psittacosis or lymphogranuloma venereum on other than clinical bases has proved extremely perplexing because of the sharing of major antigenic components by agents of this family. In limited studies, Barwell[11] and Bedson and associates[12] have found highly strain-specific skin test reactions in patients with psittacosis or L.V. when acid extract antigens prepared from these agents were used. The striking group-specificity of the skin reaction elicited by the phenol-enhanced antigens in L.V. patients is consistent with the findings of others[7-10] who showed marked dermal cross-reactivity in tests carried out in animals or patients using nonextract antigens prepared by various methods. The slight antigenic differences between strains shown in the present tests were so minor as to be of no practical consequence in attempts to obtain a species-specific diagnosis in patients. Instead, the findings showed that any of the 6 antigens, properly standardized, may be quite satisfactory for diagnosis of L.V. in man. It may be advantageous, therefore, to substitute a strain of virus nonpathogenic for man, such as the feline or mouse pneumonitis virus, for the highly virulent L.V. virus currently employed for commercial production of skin test antigen. This would reduce the hazard of infection to laboratory workers in handling the infectious material and would improve the safety of the product from the standpoint of the recipient.

SUMMARY

Skin test antigens prepared from phenol-treated suspensions of yolk sacs of embryonated eggs infected with lymphogranuloma venereum, meningopneumonitis, ornithosis, feline pneumonitis, psittacosis, or mouse pneumonitis virus all elicited positive reactions when titrated in L.V. patients. The dermal activity of certain of the heterologous viruses, shown in the titrations, was slightly less than that of the homologous L.V. virus, suggesting slight species-specificity among these agents. However, the difference was neither great enough nor sufficiently consistent to be of practical significance in providing a species-specific diagnosis in infection with these agents. It is suggested that the mouse pneumonitis or feline pneumonitis viruses, which are nonpathogenic for man, might be profitably substituted for the highly virulent L.V. virus presently used to prepare skin test antigen.

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DISTRIBUTION AND REMOVAL OF COMPONENTS CROSS-REACTIVE WITH MOUSE LIVER IN RABBIT ANTISERA AGAINST THE EHRLICH ASCITES TUMOR

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THE application of immunologic principles to the treatment of malignant tumors has occurred to several workers since the turn of the century.[1] Early attempts to use antisera made against tumor cells therapeutically yielded equivocal results; however, this field has gained considerable momentum recently, as a result of fundamental studies on tissue immunity.[2, 3] In its simplest form, the experimental approach depends on the preparation of antisera against tumor cells, the administration of such antisera to animals bearing the homologous tumor, and the regression of the tumor as a result of cytotoxic antigen-antibody reactions following specific localization of tumor antibodies at the tumor site.

With the development of radioisotope protein-labeling techniques in recent years,[4-6] the problem of localization of tissue or tumor antibodies in vivo has been extensively investigated with radioactive antibody.[7, 8] A logical extension of these principles has led to a new concept of immunologic therapy which does not necessarily rely on therapeutic effects of tumor antibody, but on radiation damage to tumors, induced by specific localization of antibody-carried radioactivity at tumor sites, which, ideally, results in localization of radioactivity at the tumor site alone, with minimal contamination of other tissues.[9]

When attempts are made to translate these principles into experimental fact, certain major problems are encountered which tend to limit greatly the amount of antibody localizing at the tumor site, and which result in undesirable deposition of antibody (and therefore radioactivity) in other tissues of the animal, notably the liver, spleen, and kidney. Such results have been predicted by similar studies on tissue antibodies,[10, 11] and greatly reduce the value of this approach. This is due to the fact that antisera prepared against tumor cells frequently involve the use of a highly complex antigenic system, resulting in the production of antibodies that cross-react with the tissue of the normal host.[12] Any method resulting in minimized cross-reactive phenomena would be of value in the solution of this problem.

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There is little doubt that the most fundamental method of attack would involve the preparation of an antigen peculiar to the tumor cell alone, and not present in host tissue. However, from an immediate practical point of view, it has seemed desirable to investigate the possibility of separation of serum components reacting with tumor cells and with normal host tissue, respectively. Newer knowledge of the diversity of antibodies, particularly to complex cellular antigens, and growing realization of the existence of serum globulins having characteristics intermediate between the principally recognized classes,[13] renders feasible the possibility of removal of cross-reactive materials in this way.

This report is concerned with such an examination of tumor antiserum. The Ehrlich mouse ascites carcinoma and its antiserum have been used as a model system.

MATERIALS AND METHODS

Mouse Liver Antigen.—Livers were removed from normal Swiss mice, placed in cold Alsever's solution and homogenized in a Potter-Elvehjem homogenizer. The suspension was centrifuged twice (35 g, 10 minutes, 0° C.), the supernates pooled, and recentrifuged (1,000 g, 20 minutes, 0° C.). The sediment from this centrifugation was washed twice with 0.85 per cent sodium chloride solution containing 0.01 per cent magnesium chloride (Mg saline) under similar conditions of centrifugation, and finally completely resuspended in Mg saline. The suspension was adjusted to 75 per cent transmission on a Lumetron colorimeter.

Preparation of Tumor Cell Antigen.—The Ehrlich mouse ascites carcinoma was carried in male Swiss mice, line transfer being effected by intraperitoneal inoculation of 0.2 ml. of ascites fluid from 7-day-old ascites tumors. Fluid was withdrawn from the peritoneal cavities of mice with 7-day-old tumors, and delivered into an equal volume of cold Alsever's solution. The cells were packed (35 g, 5 minutes, 0° C.) and washed with cold Alsever's solution until free of red blood cells. For use in complement fixation tests, the washed, packed cells were diluted with Mg saline to a concentration of 10^6 cells per milliliter suspension. Counts were made in a Petroff-Hauser counting chamber. For use as an immunizing antigen, the cells were suspended in Alsever's solution at the desired concentration.

Immunization of Rabbits.—Rabbits of both sexes (average weight, 3 kilograms) were immunized intravenously, according to the following schedule: 1×10^6 cells given in 2 doses, 3 days apart, for the first week; 2×10^6 cells and 5×10^6 cells given at similar intervals during the second and third weeks, respectively; 5×10^6 cells were administered in 2 doses, 3 days apart, during the fourth week.

Animals were bled by cardiac puncture 3 to 4 days after the final dose, and the blood allowed to clot. The individual sera were tested for antitumor titer by the quantitative (50 per cent end point) complement fixation reaction,[15] using tumor cell suspension as antigen. Sera titering at 1:1,280 or higher were pooled for fractionation.

Fractionation of Antiserum.—

Preparation of serum proteins: Pooled serum was fractionated by the low-temperature ethanol fractionation technique of Deutsch.[16] This standard procedure resolves the serum proteins into 3 main groups: albumin alpha globulin, gamma-1-beta globulins, and gamma-2 globulins. After precipitation, the first two groups were freed of alcohol by dialysis against 0.85 per cent sodium chloride, and the third group by dialysis against distilled water. The dialyzed materials were lyophilized and stored at −30° C. Fractions prepared in this way will be termed "once fractionated" (1×) fractions.

Subfractionation of gamma-1-beta and gamma-2 globulins: Once fractionated γ_2-globulin was purified by equilibrating a 1 per cent solution (ionic strength approximately 0.01) at pH 5.1 for 10 minutes at 0 to 5° C., after which the precipitate was removed by centrifugation at 0° C. The supernate was adjusted to pH 7.3 and the γ_2-globulin precipitated by adding ethanol to a final concentration of 25 per cent at −6° C. The precipitate was collected by centrifugation at this temperature, following which it was dissolved in 100 ml. distilled water, and the entire procedure repeated. The final purified γ_2-globulin was freed of alcohol by dialysis against distilled water and finally lyophilized.

Gamma-1-beta globulin, once fractionated, was dissolved in 0.85 per cent sodium chloride solution, at a concentration of approximately 1 per cent, yielding an opalescent (and slightly turbid) solution. This was adjusted to pH 7.3, equilibrated at 0 to 5° C. for 10 minutes, and the insoluble material removed by centrifugation at 0° C. The supernate was adjusted to pH 5.1 and the precipitate was collected by centrifugation at this temperature when the entire procedure was repeated. The precipitate was dissolved as before in 100 ml. 0.85 per cent sodium chloride, dried by lyophilization, and stored at −30° C.

Globulin preparations purified in this manner will be termed "thrice fractionated" (3×) fractions.

Normal serum: Pooled serum from several normal (nonimmunized) rabbits was fractionated in the same way. The normal protein fractions were used as controls in the experiments.

Complement Fixation Tests.—The quantitative (50 per cent end point) complement fixation test[15] was used. Once fractionated albumin α-globulin, γ_1-β-globulins and γ_2-globulin fractions, and thrice fractionated γ_1-β- and γ_2-globulin fractions were proportionally reconstituted to original serum concentration. All fractions were titrated against both liver and tumor antigens, end points being determined spectrophotometrically. Anticomplementary controls were run on both antigens, and also on the protein fractions.

Paper Electrophoresis of Once and Thrice Fractionated Globulins.—Samples were dissolved in 0.85 per cent sodium chloride solution. Concentrations ranged between 15 and 25 mg. protein per milliliter. Five lambda portions were applied to individual paper strips (S and S 470) with a wire loop applicator, and electrophoresis carried out in a Durrum type electrophoresis cell (Spinco Model R, Series D) in pH 8.6 veronal buffer (ionic strength 0.075) at room temperature. All fractions were run in duplicate. After electrophoresis, the strips were dried at 120° C., stained with 0.1 per cent bromophenol blue in methanol; excess stain was removed with 5 per cent acetic acid, and the color developed with ammonia. Electrophoretic patterns were recorded with an automatic integrator-densitometer (Spinco Analytrol, Model RB).

In preliminary experiments, a current of 2.5 Ma. was applied for 16 hours. In the final experiments, 15 Ma. were applied for 20 hours.

Electrophoresis of Immune Pooled Rabbit Serum.—The procedure used was exactly similar. Five lambda samples were used, and were electrophoresed as before, but at 2.5 Ma. for 16 hours.

RESULTS

Results of titration of once fractionated albumin-α-globulin, γ_2-globulin, and γ_1-β-globulin fractions from immune sera are shown in Table I. It was apparent that antitumor and antiliver activity resided in the last two globulin fractions; the albumin α-globulin fraction did not react to a significant extent with either antigenic preparation, and was consequently excluded from further consideration.

Once fractionated γ_2- and γ_1-β-globulins gave relatively high titers against tumor, these being 1:1,280 and 1:640, respectively. Against liver antigen,

titers were 1:640 and 1:1,280, respectively. Both globulin fractions, therefore, reacted strongly with these antigens and to essentially the same extent.

None of the fractions obtained from pooled normal serum reacted significantly with either antigen and was not considered further.

TABLE I. DISTRIBUTION OF CROSS-REACTIVE MATERIAL IN NORMAL RABBIT SERUM AND IN RABBIT ANTISERUM AGAINST EHRLICH ASCITES TUMOR

FRACTION TESTED		TEST ANTIGEN	
		TUMOR CELLS	LIVER PARTICULATES
Normal Serum			
Albumin α-globulin	(1×)	< 1:40	< 1:40
γ_2-globulin	(1×)	1:160	< 1:80
γ_2-globulin	(3×)	< 1:80	< 1:80
γ_1-β-globulins	(1×)	1:160	1:80
Immune Serum			
Albumin α-globulin	(1×)	< 1:40	< 1:40
γ_2-globulin	(1×)	1:1,280	1:640
γ_2-globulin	(3×)	1:1,280	1:160
γ_1-β-globulins	(1×)	1:640	1:1,280
γ_1-β-globulins	(3×)	1:80	1:80

1× = First fractionation from serum.
3× = Refractionated three times.

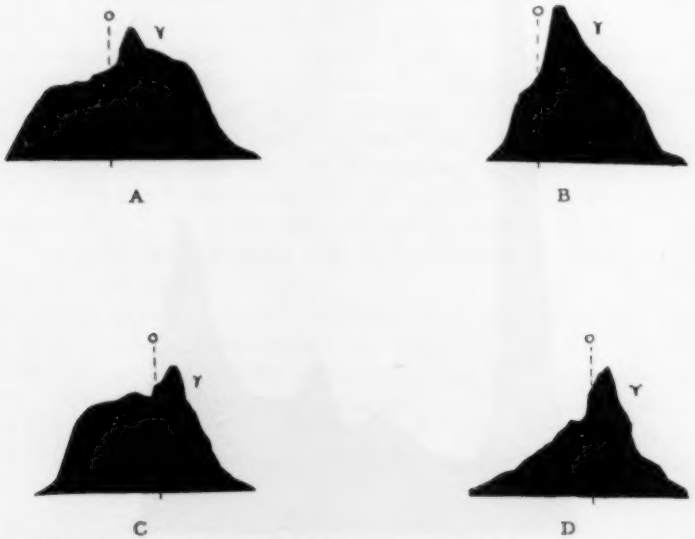

Fig. 1.—Electrophoretic changes accompanying purification of γ_2- and γ_1-β-globulins from Ehrlich ascites tumor antiserum. O = Line of origin. A, γ_2-globulin, first fractionation from serum. B, γ_2-globulin, refractionated three times. C, γ_1-β-globulins, first fractionation from serum. D, γ_1-β-globulins, refractionated three times.

Densitometer patterns from paper electrophoresis strips stained with bromphenol blue. Five lambda samples; protein concentration 20 to 25 mg. per milliliter. 15 Ma. for 20 hours in pH 8.6 veronal buffer, ionic strength 0.075.

When these globulin fractions were purified further (thrice fractionated), complement fixation tests showed a marked difference from the results with once purified material (Table I). The γ_2-globulin no longer reacted with

liver antigen to a significant extent, while preserving activity against tumor cells. The γ_1-β-globulin fraction, however, no longer reacted significantly with either antigen.

In preliminary attempts to characterize these globulin fractions by paper electrophoresis, conditions adequate for complete separation of the proteins of whole antiserum (pH 8.6 buffer, ionic strength, 0.075, 2.5 Ma., 16 hours) resulted in the formation of single broad bands with both the once fractionated globulin fractions. The limits of these bands were on both the positive and negative sides of the line of origin, but the bands themselves showed no evidence of resolution. However, when electrophoresis was repeated under conditions of higher current and for a longer time (15 Ma., 20 hours), partial resolution of these bands into two major components was obtained. In the case of both globulin fractions, a faster-moving component was present on the positive and a second component on the negative side of the line of origin.

Fig. 2.—Electrophoretic pattern of whole antiserum against Ehrlich ascites tumor. O = Line of origin. Densitometer pattern from paper electrophoresis strips stained with bromphenol blue. Five lambda whole serum, 2.5 Ma., 16 hours in pH 8.6 veronal buffer, ionic strength 0.075.

When thrice fractionated γ_2- and γ_1-β-globulin were electrophoresed under identical conditions, it was found that a major portion of the faster moving component had been removed by the purification process, while the greater portion of the slow component remained. Results of electrophoresis experiments are summarized in Fig. 1.

Results of electrophoresis of immune rabbit serum are presented in Fig. 2.

DISCUSSION

When complement fixation titers against both tumor and liver antigens were performed, findings indicated that reactivity of once fractionated serum proteins against these antigens resided in the γ_2- and γ_1-β-globulin fractions. It is known that antibodies formed by the rabbit are largely present in the γ_2-globulin fraction.[16] It was, therefore, reasoned that the antitumor titer shown by the γ_1-β-globulin fraction might well be due to contamination with γ_2-globulin, although the possibility of tumor antibodies being present in both fractions was not precluded. Conversely, complement fixation titers with once fractionated materials indicated that the reactivity of γ_2-globulin with normal mouse liver might be due to contamination with γ_1- or β-globulin, since the fraction containing these proteins showed marked cross reactivity with liver antigen. If this hypothesis was correct, purification of the two globulin fractions (γ_2 and γ_1-β) by subfractionation should have resulted in the reduction or removal of the antiliver titer of the γ_2-globulin fraction, antitumor titer being retained, and in loss of the antitumor titer of γ_1-β-globulin fraction, antiliver titer being retained.

When subjected to subfractionation, complement fixation reaction results indicated that this reasoning was indeed correct as far as γ_2-globulin was concerned. However, it was noted that γ_1-β-globulin, while losing antitumor titer, simultaneously lost its titer against mouse liver antigen. These results indicated the possibility that the once fractionated γ_1-β-globulins contained a component, certainly reactive with liver and possibly tumor antigens, that was largely removed by the purification procedure. If this was true, the antiliver activity of once fractionated γ_2-globulin might also be due to contamination with this component.

The nature of the purification accompanying subfractionation was studied by electrophoresis, in order that this possibility might be tested. It is known that when the whole serum is electrophoresed under the conditions used, it is not possible to separate γ_2- and γ_1-globulin, although β-globulin forms a distinct band due to its greater mobility in the direction of the anode. The combined γ-globulins usually migrate slightly toward the cathode, although they remain close to the line of origin. Preliminary studies on once fractionated γ_2- or γ_1-β-globulins, using comparatively low current, gave a single broad band (as described above) and, although no resolution was obtained, suggested the possibility of resolution if stronger current was used. When performed, this did in fact occur. The electrophoretic patterns obtained using once fractionated γ_2-globulin showed the presence of a γ-globulin and of a faster-moving component. Those obtained with once fractionated γ_1-β-globulin fraction showed an almost identical pattern. In the case of the γ_2-globulin fraction, the γ-globulin band indicated the presence of γ_2- (and perhaps γ_1-) globulin; this band indicated the presence of γ_1- (and possibly γ_2-) globulin in

the case of the γ_1-β-fraction. The faster-moving component in both cases occupied the same position on the paper strip. Subfractionation resulted in marked loss of the latter component in both cases.

It was, therefore, concluded that the initial reactivity of γ_2- and γ_1-β-globulin fractions with liver antigen was associated with the fast-moving component. Since the removal of this component from once fractionated γ_2-globulin did not result in loss of tumor titer, it was concluded to be not markedly reactive with tumor antigen. The antitumor titer of once fractionated γ_1-β-fraction is therefore probably due to contamination with γ_2-globulin.

The fast-moving component has been tentatively identified as a β-globulin. This component is usually completely resolved when whole serum is electrophoresed on paper, and occupies a position slightly beyond that of the fast-moving component, on the positive side of the line of origin (Fig. 2). However, 2.5 Ma. applied for 16 hours are sufficient to give this result. Data presented by Deutsch[16] show that, as fractionation of serum proceeds, the β-globulin does not resolve completely from γ_1-globulin. Cohn[14] has stated that subfractionation of β-globulin-containing fractions is difficult because of the insolubility of the precipitated material; in the subfractionation of γ_1-β-globulin described in this report, a considerable amount of insoluble material is removed.

The applications of these findings to experimental work with radioactive tumor antibody are apparent, since γ_2-globulin fractions purified in this way should result in minimized localization of radioactivity in tissues other than tumor; and should facilitate quantitative studies on tumor antibody localization in vivo, as well as attempts at therapy with such antibodies. Experiments on this aspect are being carried out at this time. It should be emphasized, however, that this approach does not in any way diminish the importance of the search for antigens that can give rise to tumor-specific antibodies.

SUMMARY

1. Serum proteins from rabbit antiserum to cells of the Ehrlich mouse ascites carcinoma have been prepared, and their reactivity with tumor and normal mouse liver antigens determined.

2. On first fractionation, both the γ_2- and γ_1-β-globulin fractions showed marked reactivity against both antigens. Purification by subfractionation resulted in removal of antiliver titer but not antitumor titer, from γ_2-globulin. Reactivity against both antigens diminished markedly in the γ_1-β-globulin fraction.

3. Electrophoretic studies of the original and the purified globulins showed the partial removal of a component, tentatively identified as a β-globulin, from both γ_2- and γ_1-β-fractions. The partial removal of this component is associated with loss of antiliver titer from both fractions.

4. Cross-reactivity with normal mouse liver of γ_2-globulin from tumor antisera can be greatly reduced by subfractionation.

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SERUM TRYPSIN DETERMINATION IN PANCREATIC DISEASE

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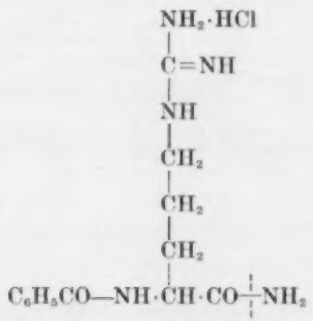
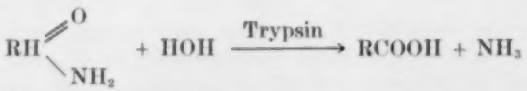
THE diagnosis of pancreatic disease, especially chronic pancreatitis and malignancy, is difficult and sometimes impossible. Serum amylase and lipase determinations, while of value in acute inflammatory states, are of little help in chronic conditions and are frequently transient in their elevations and not prognostic of the severity of the disease. The availability of synthetic polypeptide substrates with amide linkages vulnerable only to specific enzymes has enabled us to devise a simple method for the quantitative analysis of circulating serum trypsin.

The specific substrate we have utilized is α-benzoyl-l-arginine amide hydrochloride which is commercially available and has the following structural formula:



The substrate is hydrolyzed very rapidly by trypsin to yield benzoyl-l-arginine and ammonia:



To our knowledge no circulating enzyme other than trypsin will result in such hydrolysis.[1] The rate of reaction is proportional to the enzyme concentration.[2] The test is done by mixing the patient's serum with the substrate for a

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period of one hour. The ammonia formed is titrated by modification[3] of the Conway microdiffusion technique.[4] Values are expressed as units of tryptic activity. Commercial lyophilized trypsin* is used as a standard.

MATERIALS AND METHODS

Reagents.—

1. Phosphate buffer, pH 7.8.
2. Benzoyl-l-arginine hydrochloride monohydrate (BAA): 0.1 M in pH 7.8 phosphate buffer.
3. Two per cent boric acid.
4. Indicator: 5 parts of a 0.1 per cent alcoholic solution of bromcresol green; 1 part of a 0.1 per cent alcoholic solution of methyl red.
5. Saturated K_2CO_3.
6. Hydrochloric acid 0.01 N.
7. Hydrochloric acid, 0.25 N, with some indicator for soaking Conway dishes.
8. Petrolatum (for sealing).

Note: All reagents are made with freshly-boiled distilled water except number 7.

One-half milliliter of freshly drawn serum is added to 0.5 ml. of phosphate buffer and this is then added to 1.0 ml. of BAA substrate. This mixture is shaken for one hour in a "Yankee shaker" at room temperature.

While the sample is shaking, petrolatum is applied to the glass covers. One milliliter of 2 per cent boric acid solution with indicator (olive-green color) is placed in the central chamber of a Conway dish.

After shaking one hour a 0.2 ml. aliquot of the sample is deposited in the outer ring of the Conway dish. One milliliter of saturated K_2CO_3 solution is immediately pipetted into the outer ring of the dish opposite the sample aliquot taking care that they do not touch one another. The dish is tightly sealed with the glass cover, and the K_2CO_3 and sample are mixed by rotating the dish 15 times.

After one hour the central chamber of the dish is titrated with 0.01 N HCl to the original olive-green color and is compared with an extra dish containing the original 2 per cent boric acid and indicator. Subsequent titrations are compared with the one just run rather than the original dish.

All determinations including blanks are run in duplicate.

The amount of acid used in the titration is standardized against known concentrations of lyophilized crystalline trypsin, and the results are expressed as units of tryptic activity. The standard error of the determination done in duplicate is ±50 units. We have arbitrarily established 100 units as the maximum normal value.

RESULTS

A total of 58 patients were studied. Thirty-five were clinically free of pancreatic disease and were used as normals. Sixteen patients had pancreatitis, and 7 had carcinoma of the pancreas.

Of the 35 normal individuals, 8 were healthy young volunteers and 27 were hospital patients. The diagnoses and results are summarized in Table I. The average for the group was 31 units.

Sixteen patients with pancreatitis were studied (Table II). The diagnosis was proved either by laparotomy or was clinically indisputable. The average value for the group was 433 units. One patient (subject 16) with pancreatic calcinosis, insufficiency, and diabetes had no detectable amylase or trypsin.

*Tryptar, The Armour Laboratories, Kankakee, Ill.

TABLE I. SERUM TRYPSIN CONTROLS

NO.	DIAGNOSIS	UNITS
1-8	Laboratory personnel	each less than 100
9	Thyrotoxicosis	0
10	Carcinoma of tongue	0
11	Diverticulitis	0
12	Carcinoma of cecum	0
13	Postnephrectomy	0
14	Weak spells and abdominal pain	0
15	Hernia	0
16	Perforated gastric ulcer	0
17	Anorexia nervosa	0
18	Congestive failure	0
19	Postsigmoid resection	0
20	Carcinoma of rectum	0
21	Acute cholecystitis	0
22-23	Cholelithiasis	0
24-25	Dudodenal ulcer	0
26	Gallstone ileus	0
27	Cystic duct stump	0
28	Peripheral vascular disease	50
29	Abdominal pain—negative exploration	50
30	Superior mesenteric thrombosis	100
31	Postoperative hernia	120
32	Postoperative appendicitis	120
33	Chronic cholecystitis	260
34	Thorazine jaundice	80
35	Duodenal ulcer	100
	Average	31

TABLE II. SERUM TRYPSIN
PANCREATITIS

NO.	AMYLASE (N = <20)	TRYPSIN (N = < 100)	LIPASE (N = <1.0)
1	17	670	0.6
2	52	600	
3	176	600	2.3
4	1,200	580	
5	17	550	
6	136	510	9.6
7	50	500	
8	432	500	4.5
9	94	500	
10	128	450	8.0
11	200	360	
12		350	
13	8	340	0
14	270	240	
15	22	170	0
16	0	0	Pancreatic insufficiency with calcification
	Average	433	

TABLE III. SERUM TRYPSIN
CARCINOMA OF PANCREAS

NO.	AMYLASE (N = <20)	TRYPSIN (N = <100)	LIPASE (N = <1.0)
1	11	580	0.9
2	4	480	0.9
3	120	475	4.0
4	20	470	
5		300	
6	1	280	0.2
7		170	
	Average	394	

Seven patients with carcinoma of the pancreas proved by biopsy had an average value of 394 units (Table III). When available, the single highest amylase and lipase values are included in the table for purposes of comparison.

SUMMARY

Our findings suggest that in the presence of pancreatic disease there is a significant elevation of serum trypsin. This elevation is believed to result from an obstructive process in the pancreas combined with an actively secreting gland. In this series the serum trypsin determination proved a more sensitive and reliable index of pancreatic disease than either the serum amylase or lipase.

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A STUDY OF CATIONIC DETERGENT FRACTIONATION OF SERUM PROTEINS IN LYMPHOMA AND LEUKEMIA

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AN EXTENSIVE literature has accumulated in recent years regarding the use of serum protein fractionation procedures in the study of disease. Most of this work has dealt with the findings obtained using the Tiselius moving boundary electrophoresis method or, more recently, paper electrophoresis. For the past 5 years we have utilized in our laboratories a detergent fractionation method for serum proteins that was described previously.[3] This has been employed as a routine part of the laboratory examination of patients admitted to the Medical Service of the Strong Memorial Hospital. The purpose of this communication is to report our findings in a group of patients with malignant lymphoma or leukemia in whom such serum protein fractionations were carried out.

MATERIALS AND METHODS

A total of 238 detergent fractionations were done on the sera of 125 patients with malignant lymphoma or leukemia. In this group, there were 36 cases of Hodgkin's disease, 31 cases of lymphosarcoma or reticulum cell sarcoma, 18 cases of myelocytic leukemia, 12 cases of myelomonocytic leukemia, and 28 cases of lymphocytic leukemia. In each instance the diagnosis had been established by biopsy and/or examination of peripheral blood and bone marrow. Serial determinations were done on scattered patients in the group, and concomitant starch electrophoresis of serum was carried out in selected instances. These determinations were done in random fashion, with no attempt being made to select patients in particular phases of disease.

Briefly, the cationic detergent fractionation technique is based on the principle that diluted serum mixed with suitable buffers with added sodium chloride produces a quantitative precipitation of the various components of the serum when a cationic detergent (Octab) is added. Three distinct increments of turbidity can be demonstrated as the pH is increased from 6.0 to 12.0. These correspond to the alpha globulins, beta-gamma globulins, and albumin present in the serum. Photometric measurement of the turbidity in each buffer system is made for each fraction. A standard curve of the alpha globulins, total globulins, and total protein concentration is then constructed. By interpolation, the concentration of these 3 components can be determined in an unknown sample. The amount of albumin and beta-gamma globulin is determined by arithmetic calculation of the difference between total

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globulin and total protein and the difference between alpha globulin and total globulin. The cationic detergent fractionation procedure correlates closely with analysis of serum by Tiselius electrophoresis.[8]

The clinical records of the patients studied were reviewed, and in each instance an independent assessment was made of the degree of clinical activity of disease at the time that serum protein fractionation was carried out. The cases were divided into two groups, one of "active" and the other of "inactive" disease, on the basis of the clinical records. The patients grouped as "inactive" showed no or minimal objective or subjective evidence of disease, were free of signs or symptoms of hypermetabolism, were adequately nourished, and were leading essentially normal lives. Most of this group were patients in whom latent disease had been discovered by biopsy of enlarged lymph nodes or on routine examination of blood smears, while a few others represented cases of spontaneous or therapeutically induced remission in previously active disease. The group of patients classified as "active" showed unmistakable clinical signs of disease. No attempt was made to delineate subgroups within this category on the basis of severity or degree of advancement of the condition. A few patients were studied in whom determinations were made during both inactive and active phases of their illness.

Correlation of the state of clinical disease with the results of the serum protein fractionation was then carried out. Four of the 238 values were discarded because of intercurrent infection (bronchopneumonia) which has been shown to be associated with abnormalities of serum proteins. Two patients subsequently found to have lymphoma with tuberculous infection were excluded from the study. Insofar as could be ascertained, lymphoma or leukemia was the only significant disease from which the patients suffered or which might alter the serum proteins at the time the fractionations were done.

OBSERVATIONS

A grouping of the data on the basis of morphologic type of disease (lymphosarcoma, chronic myelocytic leukemia, etc.) was made. These data were then classified according to the clinical impression of disease activity. It became apparent that the common denominator with respect to abnormalities in the serum proteins was degree of clinical activity and not morphologic disease type. Our data are represented in Table I. All 5 categories of disease (Hodgkin's disease, lymphosarcoma, myelocytic leukemia, myelomonocytic leukemia, and lymphocytic leukemia) were found to exhibit similar alterations in serum proteins if the stage of clinical severity was comparable. In some entities, such as clinically inactive chronic lymphocytic leukemia, most of the determinations varied but little from normal. This group was composed of elderly patients in whom the disease was relatively benign. In other conditions, such as myelomonocytic leukemia, the protein fractionation usually displayed marked abnormality. Patients in this group were usually severely ill, and latent clinical states were uncommon. In those instances, however, in which chronic lymphocytic leukemia was clinically progressive and symptomatic, abnormal protein fractionations were the rule. Similarly, in those instances in which myelomonocytic leukemia was inactive, normal values were obtained. The classification of the cases as "inactive" and "active" was occasionally difficult, but in only a few instances were patients found to have borderline activity of their disease. These borderline patients often exhibited borderline abnormalities of the alpha globulin concentration.

The data shown in Table I indicate that no consistent alteration in total protein was found except in those patients with advanced disease associated

TABLE I. AVERAGE VALUES OBTAINED FOR SERUM PROTEIN FRACTIONATIONS IN PATIENTS WITH LEUKEMIA OR LYMPHOMA

DISEASE	CLINICAL STATE	NUMBER PATIENTS	NUMBER DETERMINATIONS	TOTAL PROTEIN (GM./100 ML.)	ALBUMIN (GM./100 ML.)	ALPHA GLOBULIN (GM./100 ML.)
Hodgkin's disease	Active	26	60	6.20	3.07	2.12
	Inactive	10	18	6.49	3.89	1.27
Lymphosarcoma	Active	22	41	6.54	3.65	1.83
	Inactive	9	17	6.45	3.86	1.20
Myelocytic leukemia	Active	8	12	6.71	3.68	1.84
	Inactive	10	16	6.55	3.85	1.11
Myelomonocytic leukemia	Active	12	14	6.81	3.35	1.76
	Inactive	2	4	6.60	3.95	1.09
Lymphocytic leukemia	Active	8	12	6.56	3.60	1.77
	Inactive	20	40	6.27	4.00	1.05

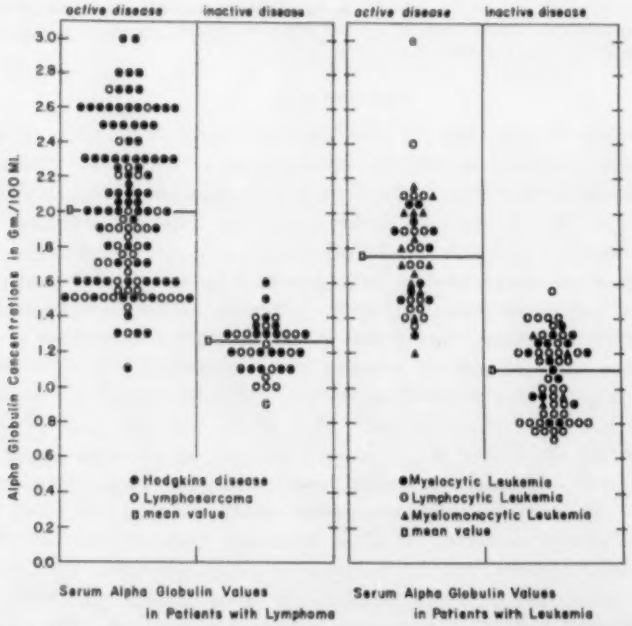

Fig. 1.—Representation of the alpha globulin values determined by cationic detergent fractionation in leukemia and lymphoma as shown by the corresponding symbols. Normal values of alpha globulin by this method range between 0.7 and 1.0 Gm./100 ml.

with severe cachexia. In these patients some decrease of total protein was present. The mean concentration of albumin was decreased in active disease and tended to parallel the degree of general body wasting.

The most significant and striking alterations were noted in the alpha globulin fraction. A marked elevation was found in "active" disease whereas essentially normal or minimally elevated values were found in patients with "inactive" disease. This is clearly shown in Fig. 1 which illustrates in composite fashion the grouping and range of values obtained for alpha globulin in the various conditions under study.

It will be noted that in the "active" disease states, few values for alpha globulin less than 1.4 Gm./100 ml. were obtained, and that practically all of the values in "inactive" disease fell below the 1.4 Gm./100 ml. line. The only significant exception to this generalization was noted in terminal patients. In these patients, previously elevated values were frequently observed to fall to borderline or only slightly elevated levels.

The results of serial studies are of particular interest in those patients who showed progression of clinical disease from an inactive to an active state. This is illustrated by the case of L. W. (Table II).

TABLE II. VALUES FOR SERIAL SERUM PROTEIN FRACTIONATIONS OBTAINED IN A CASE OF LYMPHOSARCOMA (SEE TEXT, CASE 1)

DATE	TOTAL PROTEIN (GM./100 ML.)	ALBUMIN (GM./100 ML.)	ALPHA GLOBULIN (GM./100 ML.)	COMMENT
12- 2-53	6.9	3.65	1.0	Inactive disease
4-26-55	6.9	3.65	1.9	Asymptomatic, gradually increasing lymphadenopathy
12-12-56	7.3	3.4	2.4	Active, symptomatic disease
7-24-57	6.3	2.75	1.95	Recurrent active disease following remission

CASE 1.—The patient was a 47-year-old white woman first seen on Oct. 28, 1952, with a 2 months' history of mild asymptomatic lymphadenopathy. A biopsy of a supraclavicular node at this time showed only hyperplasia. A biopsy of an epitrochlear node in December, 1952, 1 month later, revealed a more atypical but nondiagnostic hyperplasia. The patient remained asymptomatic with persistent lymphadenopathy over the ensuing year. Protein fractionation on Dec. 2, 1953, showed total protein 6.9 Gm./100 ml., albumin 3.65 Gm./100 ml., and alpha globulin 1.0 Gm./100 ml. The patient was seen at intervals during the next year and continued to remain asymptomatic. Early in 1955, gradual enlargement of the right axillary nodes was noted, together with the development of a small mass in the right breast. The breast lesion was biopsied in April, 1955, revealing lymphoid infiltration suggestive of lymphoma. Protein fractionation on April 26, 1955, showed total protein 6.9 Gm./100 ml., albumin 3.65 Gm./100 ml., and alpha globulin 1.9 Gm./100 ml. The patient then developed a palatal mass with a loss of hearing in the left ear. X-ray therapy was directed at the palatal area during the next two months with reduction of the tumor mass. Between September and October, 1955, marked generalized lymphadenopathy developed. The patient now displayed systemic symptoms of active disease for the first time. A biopsy of a right epitrochlear node in October, 1955, revealed a lymphoblastic lymphosarcoma. Two courses of local x-radiation were administered over the next 6 months with decrease in size of the lesions. By December, 1956, adenopathy recurred, and the patient was hospitalized for symptoms of dyspnea and cough. Examination revealed a left hydrothorax and mediastinal lymphadenopathy. The protein fractionation on Dec. 12, 1956, showed total protein 7.3 Gm./100 ml., albumin 3.4 Gm./100 ml., and

alpha globulin 2.4 Gm./100 ml. The patient was again treated with x-ray, and a good clinical response was obtained. In July, 1957, massive lymphadenopathy with associated symptoms recurred. The protein fractionation on July 24, 1957, showed total protein 6.3 Gm./100 ml., albumin 2.75 Gm./100 ml., and alpha globulin 1.95 Gm. per 100 ml.

During the period of observation in this patient, the total protein and albumin values remained essentially the same while the alpha globulins rose from normal to more than twice the original figure. It is noteworthy that the rise in alpha globulins preceded by 5 months the development of frankly progressive clinical disease.

On several other occasions in our experience, elevated alpha globulin concentrations have anticipated the development or reactivation of florid clinical disease. An example of this is seen in our study of a 30-year-old man. A diagnosis of lymphosarcoma was reliably established by biopsy in 1945. In 1952, the patient was treated with x-ray for enlarged abdominal nodes which had caused biliary obstruction. A complete remission resulted. The alpha globulin concentrations were found to be elevated in September, 1953, and June, 1954, to 1.65 Gm./100 ml. and 1.7 Gm./100 ml., respectively, during the period (1953-1955) when the patient was completely asymptomatic. He was accepted for military service early in 1954. In February, 1955, his disease became clinically active. He expired 9 months later with widespread lymphosarcoma.

In contrast to these cases, we may cite our experience with 8 other cases of lymphoma with no clinical evidence of active disease. All of these patients have had normal alpha globulin concentrations for more than 3 years.

Several instances have been observed in which a fall of alpha globulin concentration from previously elevated values occurred concomitantly with therapeutically induced remission of the disease. This is shown in the case of C. C. (Table III).

TABLE III. VALUES FOR SERIAL SERUM PROTEIN FRACTIONATIONS OBTAINED IN A CASE OF HODGKIN'S DISEASE (SEE TEXT, CASE 2)

DATE	TOTAL PROTEIN (GM./100 ML.)	ALBUMIN (GM./100 ML.)	ALPHA GLOBULIN (GM./100 ML.)	COMMENT
11-17-53	5.9	2.75	1.0	Therapeutically induced remission
1-21-54	5.7	3.3	1.6	Reactivation of disease
3-22-54	5.3	2.0	2.6	Severely ill
3-29-54	5.3	2.7	1.5	Therapeutically induced remission
4-12-54	6.0	3.65	1.2	Further improvement
7-26-54	4.7	2.3	1.6	Relapse with rapid deterioration

CASE 2.—The patient was a 59-year-old white man first seen in November, 1953. A diagnosis of Hodgkin's disease had been made 3 years previously by means of a cervical lymph node biopsy. He had been intermittently treated with x-ray. One month prior to admission he had received triethylene melamine for a reactivation of disease and obtained a good response. A protein fractionation on Nov. 17, 1953, showed total protein 5.9 Gm./100 ml., albumin 2.75 Gm./100 ml., and alpha globulin 1.0 Gm./100 ml. During the ensuing 2

months the patient became increasingly symptomatic. On Jan. 21, 1954, his serum showed total protein 5.7 Gm./100 ml., albumin 3.3 Gm./100 ml., and alpha globulin 1.6 Gm./100 ml. He received x-ray therapy with transient improvement. He then had a relapse and was admitted to the hospital in March, 1954. On March 22, 1954, serum analysis showed total protein 5.3 Gm./100 ml., albumin 2.0 Gm./100 ml., and alpha globulin 2.6 Gm./100 ml. He was given nitrogen mustard during the next week with relief of symptoms. On March 29, 1954, he showed total protein 5.3 Gm./100 ml., albumin 2.7 Gm./100 ml., and alpha globulin 1.5 Gm./100 ml. Further clinical improvement occurred over the ensuing 2 months. On April 12, 1954, his fractionation showed total protein 6.0 Gm./100 ml., albumin 3.65 Gm./100 ml., and alpha globulin 1.2 Gm./100 ml. In the ensuing month the patient began to fail rapidly. On July 26, 1954, his serum fractionation showed total protein 4.7 Gm./100 ml., albumin 2.3 Gm./100 ml., and alpha globulin 1.6 Gm./100 ml. The patient died 2 weeks later. It is to be noted that the alpha globulin concentration fell significantly as the patient's clinical condition improved and mirrored the patient's condition accurately as reactivation occurred.

DISCUSSION

In general, our observations are in agreement with those previously reported by others. In 1947, Nitshe and Cohen[6] described their findings in a group of 26 patients with Hodgkin's disease, 15 with myelocytic leukemia, and 12 with lymphocytic leukemia, studied with fractionation of serum albumin and globulin using a methyl alcohol precipitation technique. They found no significant alteration from normal in the total protein but observed generally decreased albumin and increased globulin fractions in cases of Hodgkin's disease and myelocytic leukemia. These authors noted that abnormalities in the fractionation were more pronounced as disease advanced and that some change toward normal occurred after radiation therapy.

Rottino and co-workers,[8] in 1948, reported electrophoretic studies on the sera of 27 patients with Hodgkin's disease. These workers concluded that the patterns obtained were nonspecific for the disease. Three general patterns were seen: type A (11 patients) with an essentially normal electrophoretic pattern, from patients in good nutrition and asymptomatic; type B (9 patients) characterized by decreased albumin and increased alpha globulin concentrations, seen in patients who had terminal disease with symptoms of hypermetabolism; and type C (7 patients) characterized by increased gamma globulin concentrations from patients exhibiting an intermediate clinical picture.

Mider and co-workers,[5] in 1950, reported their results in an electrophoretic study of the serum protein of 222 cancer patients, including 36 with lymphoma or leukemia. They found that the albumin concentration decreased progressively as the disease advanced, with a significant decrease of this fraction found early in the disease. The total globulin concentration was increased with the alpha globulin increased proportionately. The beta and gamma fractions were less elevated while the fibrinogen concentration increased disproportionately. In late cancer, the globulins were significantly increased as the malignancy progressed. No differences in this general pattern were found on comparing values from cases of lymphoma with those from cases of carcinoma of the breast, colon, or stomach.

Brown and associates,[2] Peterman and associates,[7] Tischendorf and Hartmann,[11] Arends and co-workers,[1] Rundles and associates,[9] Jencks and associates,[4] and Sunderman and Sunderman[10] have reported similar findings in electrophoretic studies of the sera of patients with lymphoma and/or leukemia.

It is evident from our work and that of other investigators that several points can be made: (1) There is no serum protein pattern characteristic of any of the malignant lymphomas or leukemias. (2) The specific alterations consist of a decrease in the albumin concentration and increase in the alpha globulin concentration, with less consistent variations observed in the other fractions. (3) The degree of abnormality in these protein fractions bears a relationship to the severity of the clinical disease.

Our observations indicate that the determination of the alpha globulin fraction of the serum is the most useful indicator of the progression of disease. It should be emphasized that the clinical activity of the disease process is more important than the pathologic entity. Our data further suggest that the determination of the alpha globulin concentration of serum may serve to predict the immediate future clinical course of the patient. A rise in concentration of this fraction appears to anticipate clinical exacerbation of disease. An elevated alpha globulin value in a patient with known but inactive or remittent leukemia or lymphoma has usually been an ominous prognostic sign. On the other hand, a succession of relatively normal values of alpha globulin has indicated latent disease. A sustained lowering of the alpha globulin values following therapeutically induced or spontaneous remission in these conditions has been a favorable indicator of the patient's immediate future course.

The use of the cationic detergent fractionation technique has several advantages over other methods of protein analysis. As noted previously,[3] the procedure yields values closely comparable to those obtained using the Tiselius electrophoresis technique, although the same detailed separation of the serum proteins is not possible. The detergent fractionation technique is a simple, rapid and inexpensive analytic procedure. It is superior to conventional sodium sulfate fractionation. Jencks and his co-workers[4] have emphasized that the usual salt precipitation technique for separating albumin from globulin is unsatisfactory since most of the alpha globulin can be shown to reside in the albumin fraction. Hence, a fall in serum albumin associated with a rise in alpha globulin may not be detected by sodium sulfate fractionation. In view of the demonstrated importance of these two components in our evaluation of this group of diseases, this point assumes increased significance.

SUMMARY

1. Two hundred thirty-four detergent protein fractionation studies on the sera of 125 patients with malignant lymphoma or leukemia are presented and correlated with the clinical activity of the patient's disease.

2. The alpha globulin fraction was found to reflect accurately the patient's clinical condition. This fraction was increased during the acute disease and

normal or only slightly elevated in inactive disease. In several instances, an elevation of alpha globulin anticipated the onset of active disease.

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CHLORIDE, POTASSIUM, AND ALDOSTERONE EXCRETION IN A SODIUM-RETAINING PATIENT (HEPATIC CIRRHOSIS) DURING FORCED NATRIURESIS

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DURING the early stages, at least, of an acutely-induced rise in electrolyte excretion in sodium-retaining patients, the principal cation is frequently observed to be potassium. As examples which by no means exhaust the literature, Eichna and associates[1] noted that potassium rather than sodium excretion increased following administration of Digoxin to a patient with congestive heart failure. Metcoff, Gordillo, and Antonowicz[2] described a predominant increase in potassium excretion in 4 nephrotic children following infusion of either Diamox or sodium para-aminohippurate. The anion (chloride, bicarbonate, or aminohippurate) was different in each of the 3 circumstances noted. Since sodium-retaining states such as congestive failure and nephrosis are generally associated with markedly increased urinary excretion rates of the adrenal salt-active steroid, aldosterone,[3-5] and since primary aldosteronism is characterized, among other things, by potassium depletion,[6] it seemed reasonable to attribute the acute kaliuresis in the experiments noted above to the effects of an elevated rate of aldosterone secretion. According to this hypothesis the rate of potassium excretion by a given kidney (assuming a constant state of body electrolytes) depends upon the simultaneous magnitudes of aldosterone production and of sodium delivery to the tubular site of potassium-sodium exchange.

The present study was, therefore, undertaken (1) to extend to salt-retaining cirrhotic patients the type of experiment described for congestive failure and nephrotic patients, particularly since cirrhotics have also been reported to exhibit high urinary aldosterone excretion rates[3]; (2) to extend the period of observation of the enhanced rate of urinary electrolyte excretion; and (3) to determine whether the potassium losses predicted by the hypothesis would detectably affect either aldosterone excretion or any of the secondary evidences of increased aldosterone activity. This last observation was suggested by reports of an influence on aldosterone excretion of changes in body potassium balance.[5, 7]

MATERIALS AND METHODS

A 59-year-old patient with ascites secondary to Laennec's cirrhosis was selected for study. He responded poorly to diuretics and salt-restriction so that occasional paracenteses

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were necessary to relieve the continuously forming ascites. He had no evidence of renal disease. After 10 days of equilibration in a metabolism ward on a diet containing approximately 30 mM. of sodium and 60 mM. of potassium per 24 hours, an infusion of sodium sulfate (100 mM./L.) was started at 0.7 ml. per minute. An additional 100 ml. of the same solution was given during the first hour as a priming dose. The infusion was continued by a pump at 0.7 ml. per minute, without interruption for 3 days. With a comparatively small increase in plasma sulfate concentration, most of the increase in filtered sulfate is excreted in the urine, obligating correspondingly increased amounts of cation, mostly sodium and potassium, in the urine. Although some sulfate with isosmotic equivalent volumes of water are stored in the process of elevating the blood sulfate concentration, the amounts so stored and any resulting depressant effect of this fluid volume expansion on aldosterone excretion must be relatively small. Venous blood samples and urine and feces were collected on the 3 days preceding, during, and on the 3 days following the infusion.

Sodium and potassium were measured in plasma, urine, and feces by internal standard flame photometry; chloride was measured in plasma and urine by the iodometric method of Van Slyke and Hiller.[8] Urinary steroid salt-retentive activity was measured in terms of equivalent desoxycorticosterone activity by a rat bio-assay technique modified from that of Johnson.[9] The method may be described briefly as follows. Rats, adrenalectomized at least one week prior to the assay, were tested for evidence of adrenal insufficiency by weight loss on saline deprivation. No animal losing less than 5 Gm. on 18 hours of salt deprivation was used in the assay. The day preceding a test, water was exchanged for saline to ensure some degree of sodium depletion. The day of the test 5 ml. of 0.9 per cent saline was injected intraperitoneally. One hour later, a urine sample representing the control excretion value for that animal was collected and 0.1 ml. absolute alcohol was injected subcutaneously, either as a blank or containing desoxycorticosterone standards or urine extract prepared by the method of Singer and Venning.[10] After another hour, a second urine sample (test) was obtained and the potassium-sodium (K-Na) ratios on each urine sample were measured. The test and control K-Na ratios of each group of 6 to 10 rats receiving a given injection were averaged, and the difference between the mean test and control ratios, divided by the control, was the response of the group. By inspection it was determined that the response plotted against the $\frac{2}{3}$ power of the desoxycorticosterone dose was approximately linear between 0 and 60 μg per rat with a small positive intercept due to the blank. Dosage was adjusted on a 300 gram rat basis. We do not consider the assay results by this method accurate to better than a factor of 2.0.

RESULTS AND DISCUSSION

The results are illustrated in Fig. 1. In this ascitic cirrhotic patient, potassium appeared abruptly in large amounts at the start of the sodium sulfate infusion (fourth day). The 3 excretion rate figures on this and the seventh day when the infusion was stopped represent the mean rates per minute during the first 4, second 4, and final 16 hours of these days. Sodium excretion did not increase until several hours following the onset of the kaliuresis. The distinct negative potassium balance during the 3 day test attained a cumulative total of approximately 200 mM. and was associated with a decrease in plasma potassium of about 1.0 mM./L. In retrospect, the experiment is obviously too short to estimate how long potassium losses would have continued. Chloride excretion decreased from control values of 1 to 3 mM. per 24 hours to virtually 0 (less than 0.5) at the end of and during the 3 days following the infusion. The significance of the decrease in chloride excretion cannot be interpreted in any

simple fashion. Heavy sulfate loading may be accompanied by large increases in chloride excretion.[11] Schwartz, Smith, and Winkler[12] observed a decrease in chloride excretion during moderate sulfate loading in dogs even though plasma chloride concentration was increasing. Filtration rate, however, tended to decrease so that the actual tubular loading terms, particularly for chloride, are ambiguous.

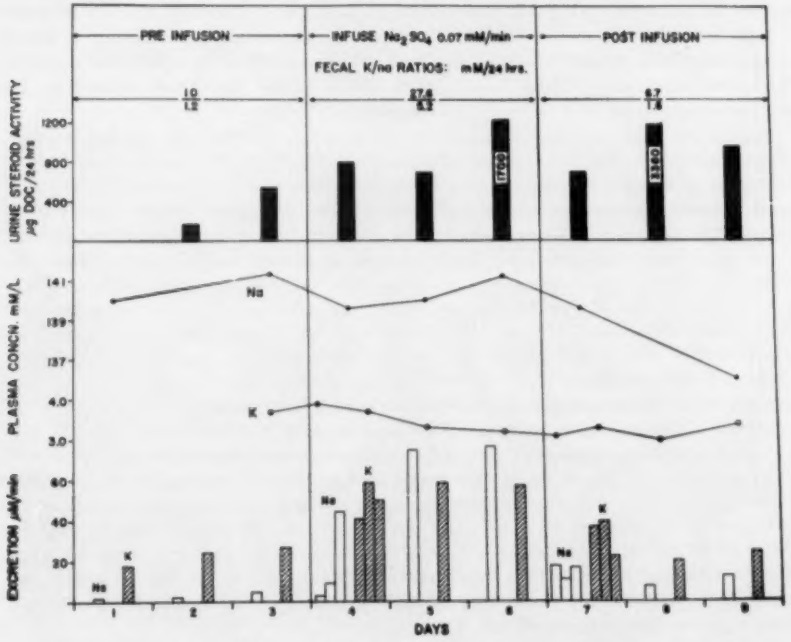

Fig. 1.—Effects of sodium sulfate infusion on sodium and potassium excretion, plasma sodium and potassium concentrations, urinary salt-active steroid excretion, and fecal potassium-sodium ratios in a patient with cirrhosis and ascites. The tripartite bar graphs of sodium and potassium excretion on the fourth and seventh days represent mean excretion rates during the first 4, second 4, and final 16 hours, respectively.

No evidence of a gross decrease in aldosterone excretion occurred either in the presence of relatively large amounts of sodium (0.07 mM. per minute) in the urine or as a result of a marked negative potassium balance and hypokalemia. This conclusion is, of course, qualified by the wide margin of error of the method of assay. Supporting the direct evidence of sustained high aldosterone activity are the extremely low urine chloride excretion and the high stool K-Na ratios. Our experiment leads to conclusions different from those of Laragh and Stoerk[13] with respect to the effect of moderate potassium depletion upon aldosterone excretion in the presence of sodium retention. These investigators reported a tenfold decrease in aldosterone excretion following potassium loss resulting from mercurial diuresis. Their low figure appears to be the report of a single day's analysis, and as such is consistent, at least in part, either with

what we interpret to be random variation in the method (an anomalously high value was observed on another day of their study) or with acute effects of Mercuhydrin-induced alterations in renal function. The evidence of our experiment accords with the views of Bartter[5] and others that the salt-retaining state (ascites formation in this patient) constitutes the prepotent stimulus to aldosterone secretion in man, with the result that continuation of aldosterone production in the presence of an ample supply of sodium as the substrate for cation exchange in the renal tubules results in large potassium losses.

SUMMARY

Sodium sulfate was infused at 0.07 mM. per minute for 3 days in a patient who was retaining sodium chloride and forming ascitic fluid secondary to Laennec's cirrhosis. The following results were noted.

1. Urinary sodium excretion increased probably because of increased sulfate excretion.

2. Potassium excretion increased markedly, accompanied by a negative potassium balance and decreased serum potassium concentration.

3. Urinary chloride excretion decreased.

4. Fecal potassium-sodium ratios decreased by about one-half, which is not considered significant.

5. Urinary aldosterone excretion did not decrease significantly.

The data are interpreted as supporting the view that aldosterone excretion responds to some complex physiologic state of the body and, to a lesser degree, to either urinary sodium content, plasma potassium concentration, or body potassium content.

We gratefully acknowledge our indebtedness to Dr. Nicholas Mazarella, Dr. Guillermo Morales, Miss Cornelia Culpeper, and Mr. Leonard Beier for their assistance with this study.

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THE ANEMIA ASSOCIATED WITH RENAL DISEASE

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THE association of anemia with disease of the kidneys has been noted from the time of Bright's original observations.[1] The anemia has been characterized as normocytic and normochromic and to be refractory to the usual methods of treatment. More recently further investigation has been possible with the use of isotopic labeling procedures.[2-7] This paper reports our findings using these methods in a group of patients with both acute and chronic renal disease of differing etiology.

MATERIALS AND METHODS

The subjects used in this study were all carefully selected from a much larger group of patients with renal disease, who were under observation in this department. They were all men. None had received hematinics or other active treatment prior to study. The criteria for inclusion were the presence of anemia for which no other cause was found, or the presence of markedly impaired renal function. Those with a hemoglobin of 12 grams per cent or less are included in Group 3. Those with a hemoglobin greater than 12 grams per cent are in Group 2. Additional patients not included elsewhere in this study were used to relate blood urea nitrogen and hemoglobin. Both these values were stable at the time of selection. Detailed renal function tests were done on all but have not been included as the blood urea nitrogen and serum creatinine serve as an adequate guide to the level of renal insufficiency. In the patients with pyelonephritis, there were none of the usual clinical or laboratory features associated with an active infectious process. Fever, pyuria, and leukocytosis were all absent and urine cultures were frequently sterile. Biopsy or autopsy evidence in many indicated a very slowly progressive chronic process, and until such time as renal failure became severe, general health was well maintained. It was believed on these grounds that the "anemia of infection" could probably be excluded. In every instance the patients have personally been followed carefully for a minimum of 3 months, but in many cases for 1 to 2 years. No other cause for anemia has appeared. In the 15 patients in Group 3 with hemoglobins below 12 grams per cent, 6 have been autopsied and, of the remainder, 5 have had percutaneous renal biopsy to confirm their diagnoses, at some time during observation. Stools for occult blood were performed repeatedly in every instance and were invariably negative.

The control group of 10 men consisted of 5 healthy doctors on the intern staff of the hospital and 5 patients who were selected because of the absence of any disorder which would affect the hemopoietic system. The data from each group of 5 have been pooled as they were essentially similar.

Hemoglobin was measured colorimetrically as cyanmethemoglobin.[8] Wintrobe tubes were used for hematocrit determinations. They were centrifuged for 55 minutes at 3,000

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r.p.m. in a centrifuge with a radius of 15 cm. from tube tip to spindle. The values obtained were corrected for trapped plasma according to the determined values given by Chaplin and Mollison.[9] Reticulocyte counts were made by counting 1,000 red cells. Aspiration bone marrows were obtained from the sternum. Three hundred cells were counted. Fecal urobilinogen was determined on a minimum of two 4-day pools.[10] The beginning and end of the 8 days were noted using a carmine marker. All collections were carried out on a metabolic unit.* Serum iron was measured using the method as described by Powell[11] and iron binding capacity by the method of Rath and Finch.[12] Samples were collected using specially cleaned tubes and needles without the use of a syringe.†

Fe^{59} as ferrous citrate was obtained from Abbott Laboratories and was of high specific activity in the order of 1.5 μc per microgram. Ten to fifteen microcuries was given to each patient. The weight of iron varied from 6 to 14 μg, the majority being 10 μg. The studies using radioiron were all started between 8:30 and 9:00 A.M. with the subjects in the postabsorptive state with free access to water. At least 20 ml. of the subjects' plasma was incubated with the radioiron for 30 minutes at room temperature with frequent mixing. At the end of this time approximately 5 ml. of Evans blue 0.5 per cent solution was injected intravenously followed by the plasma containing bound radioiron after removal of 1 ml. for dilution in 500 ml. saline as a standard. The amounts contained in the 3 syringes were determined gravimetrically on an accurate torsion balance. Syringes were weighed full and then after delivery. The same needle attached to a stopcock was used to inject both Evans blue and plasma. This was washed clear finally with 10 c.c. of saline. Samples were removed at intervals of 10, 20, 30, 40, 60, 90, 180, 240, and 360 minutes following injection from the opposite arm. All blood samples were heparinized. During the first two and one half hours the subjects were reclining in the laboratory. Subsequently they were allowed to return to the ward. The following day and daily thereafter blood samples were taken until the amount of radioiron appearing in the circulating erythrocytes had reached a plateau. In several instances counting extended as long as 3 to 4 weeks with less frequent sampling during the latter part of this time. The standard was counted simultaneously with the unknowns. To measure the rate of disappearance of injected radioiron, 3 ml. samples of plasma were counted in duplicate in a well-type scintillation counter with a thallium activated sodium iodide crystal.‡ Net counts per minute were then plotted semilogarithmically against time. The amount of radioactivity appearing in the blood after the day of injection was determined by counting 3 ml. samples of whole blood in the same way. Plasma activity was always checked separately; however, by 24 hours after injection it was almost negligible and by 48 hours was zero. The total circulating activity of the red cell mass was determined by calculating the activity in 1 ml. of red blood cells after correction for trapped plasma and then multiplying by the red cell volume. A minimum of 10,000 counts were recorded on all radioactive samples reported.

To determine plasma volume the dilution of injected Evans blue was determined on samples obtained at 10, 20, and 30 minutes. Three milliliter plasma samples were diluted with 3 ml. 0.9 per cent sodium chloride and the optical density read at 620 mμ in a Beckman Model B spectrophotometer. A standard curve was made by diluting the dye in normal plasma. The same batch of dye was used throughhout. A plasma blank obtained immediately prior to injection was used to set the instrument to 100 per cent transmission. Dye concentrations obtained were plotted against time on semilog paper. A straight line was invariably obtained. This was extrapolated back to zero time and this concentration was used for calculation of plasma volume. For correction of the discrepancy between peripheral and whole body hematocrit the factor of 0.91 was employed.[13]

$$\text{Plasma volume (P.V.)} = \frac{\mu\text{g dye injected}}{\text{dye conc. at zero time in } \mu\text{g/ml.}}$$

*Clinical Investigation Unit, Queen Mary Veterans' Hospital, Montreal.

†These determinations were kindly done by the Department of Hematology, Royal Victoria Hospital, Montreal.

‡Manufactured by Electronic Associates, Willowdale, Ontario.

$$\text{Blood volume} = \text{P.V.} \times \frac{100}{100 - (\text{hematocrit corrected for trapped plasma} \times 0.91)}.$$

For calculation of the plasma iron turnover rate and other indices of iron metabolism, the formulas derived by Huff and co-workers were used.[14]

In the determination of red cell survival, 40 ml. of blood was mixed with 5 ml. of A.C.D. solution containing 150 μc of radiochromium as sodium chromate and incubated at room temperature for 30 minutes, with periodic gentle mixing. Following incubation the cells were washed twice in sterile isotonic saline, made up to volume with saline solution and reinjected. After 48 hours a sample was removed and counted in the same manner as the Fe^{59} samples. The activity in 1 ml. of red cells in this specimen represented 100 per cent activity. Subsequently samples were removed once or more often twice weekly. In all instances, with one exception (W. L.) to be described later, the hematocrit remained constant during the sampling period. The standard was counted at the same time as subsequent samples to eliminate variations in counting efficiency and correction for radioactive decay.

Blood urea nitrogen (B.U.N.) was determined by Archibald's method.[15] Serum creatinine was measured after elution from Lloyd's reagent.[16] Twenty-six normal subjects showed a range from 0.37 to 1.12 mg. per cent with a mean of 0.84 ± 0.14.

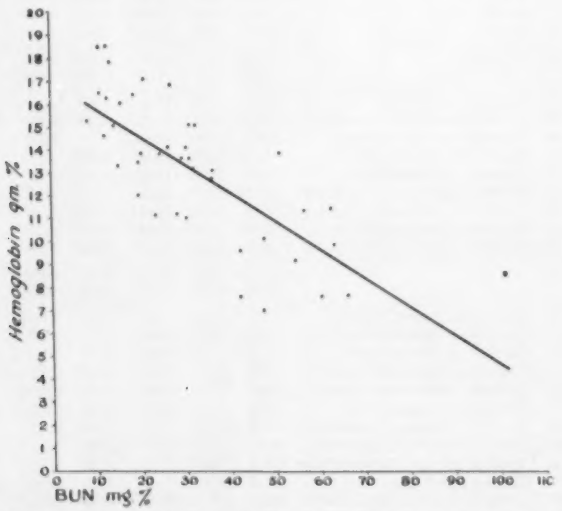

Fig. 1.—Relationship between blood urea nitrogen and hemoglobin ($r = 0.756$, $p < 0.001$).

RESULTS

The individual values with means are shown in Table I. There was a steady fall in the mean values for hemoglobin in each group from 15.3 Gm. per cent in the controls to 13.6 Gm. per cent in Group 2 and 9.8 Gm. per cent in Group 3. This fall was associated with evidence of increasingly severe renal disease, the mean blood urea nitrogen (B.U.N.) rising from 12.2 mg. per cent to 30.5 mg. per cent and 54.9 mg. per cent in each group. That a fairly close relationship existed between renal function as measured by B.U.N. and the level of hemoglobin is shown in Fig. 1. Each dot represents a single reading on one

TABLE

GROUP*	PATIENT	AGE	DIAGNOSIS	B.U.N. (MG.%)	CREAT. (MG.%)	HB. (GM.%)	RED CELL COUNT (MILLIONS/C.MM.)	HMCT. (%)	RETICS. (%)	MCV (CU. μ)	MCH ($\mu\mu$G)
I	R. L.	55	Osteoarthritis	12.8	-	13.9	4.60	43.0	1.7	93.0	30.0
I	N. B.	66	Healed peptic ulcer	10.5	-	12.7	4.23	40.0	1.2	95.0	30.0
I	B. L.	53	Osteoarthritis	12.8	-	14.9	4.70	46.0	0.7	100.0	31.6
I	R. R.	52	Coronary artery disease	14.6	-	17.5	5.22	51.0	0.4	98.0	33.6
I	B. G.	63	Coronary artery disease	7.5	-	16.3	4.95	47.0	0.2	95.0	33.0
I	D. F.	32	Staff	12.5	0.88	14.2	4.54	43.0	0.8	95.0	31.0
I	R. S.	30	Staff	17.7	0.92	15.0	4.72	46.5	1.2	100.0	31.5
I	N. F.	30	Staff	9.4	0.96	16.8	5.00	49.0	1.7	95.0	32.0
I	J. G.	24	Staff	8.8	0.92	16.5	5.41	47.7	0.8	90.0	30.0
I	W. B.	32	Staff	12.0	1.07	14.9	4.80	45.8	0.7	94.0	30.0
	Mean	43.7		11.9		15.3	4.81	45.9	0.9	95.5	31.2
	± S.D.			4.5		1.48	0.36	3.21	0.5		
II	F. D.	51	Chronic pyelonephritis	31.0	3.90	13.2	4.03	40.5	1.7	100.0	32.0
II	O. F.	46	Acute glomerulonephritis	19.0	1.40	13.9	4.24	38.0	1.0	90.0	33.0
II	A. M.	46	Chronic pyelonephritis	24.0	2.80	13.9	-	43.3	-	-	-
II	A. C.	52	Essential hypertension	8.0	1.96	15.2	4.40	44.0	0.4	100.0	32.0
II	H. D.	60	Chronic pyelonephritis	34.0	1.90	13.3	4.34	38.0	0.5	88.0	31.0
II	A. P.	31	Chronic glomerulonephritis	35.4	3.00	14.1	4.60	40.0	2.4	86.0	30.0
II	E. L.	41	Chronic pyelonephritis	51.4	4.00	13.9	4.46	41.0	1.7	93.0	31.1
II	A. R.	63	Chronic pyelonephritis	35.8	2.70	12.9	4.15	39.0	1.0	95.0	31.0
II	A. L.	60	Chronic pyelonephritis	36.6	3.76	12.3	3.74	37.0	0.8	92.0	32.0
	Mean	50		30.5	2.82	13.6	4.25	40.0	1.2	93.0	31.5
	± S.D.			12.3	0.93	0.82	0.85	2.39	0.68		
III	N. L.	62	Essential hypertension	42.0	3.40	7.6	2.90	24.8	0.8	85.5	26.2
III	L. S.	64	Chronic pyelonephritis	42.0	3.60	9.7	3.20	31.0	2.7	96.8	32.5
III	F. H.	38	Chronic pyelonephritis	60.0	5.00	9.2	3.10	27.1	1.9	87.5	29.7
III	W. L.	63	Chronic pyelonephritis	60.0	6.10	7.6	2.40	24.7	2.4	95.0	28.3
III	M. D.	57	Chronic pyelonephritis	56.0	3.50	11.3	3.60	35.9	1.3	99.8	31.8
III	A. D.	54	Chronic pyelonephritis	35.2	3.20	11.8	3.85	37.1	0.6	96.4	31.3
III	C. W.	36	Diabetic glomerulosclerosis	47.0	3.90	7.0	2.10	20.7	5.2	98.0	33.0
III	O. F.	46	Acute glomerulonephritis	28.0	2.60	11.2	3.90	31.0	0.5	79.0	28.7
III	J. P.	34	Chronic glomerulonephritis	91.0	10.00	10.8	3.86	30.0	1.0	84.0	28.4
III	J. L.	76	Chronic pyelonephritis	51.4	4.00	8.6	2.80	25.0	1.5	89.0	30.7
III	M. d'A.	29	Acute glomerulonephritis	65.0	2.01	11.8	4.15	30.0	1.5	81.0	27.5
III	A. P.	69	Acute glomerulonephritis	40.0	3.60	10.2	3.35	31.0	0.8	90.0	30.0
III	G. C.	63	Chronic glomerulonephritis and chronic pyelonephritis	31.2	3.29	11.6	3.40	33.0	0.1	93.0	32.0
III	E. S.	39	Subacute glomerulonephritis	35.2	4.72	11.1	3.63	33.0	0.1	91.0	33.0
III	R. A.	35	Chronic pyelonephritis	140.0	19.1	8.0	2.41	24.0	1.2	100.0	33.0
	Mean	51		54.9	5.2	9.8	3.24	29.2	1.4	91.0	30.4
	± S.D.			28.5	4.27	1.7	0.6	4.69	1.27		
Group	I vs. II					0.01	0.01	0.001	0.3		
Group	I vs. III		p = less than			0.001	0.001	0.001	0.3		
Group	II vs. III					0.001	0.001	0.001	0.7		

*Group 1 = normal subjects. Group 2 = patients with renal disease with hemoglobin above 12 Gm. per cent. Group 3 = patients with renal disease with hemoglobin under 12 Gm. per cent.

I

MCHC (%)	PLASMA VOL. (ML./KG.)	R.B.C. VOL. (ML./KG.)	BLOOD VOL. (ML./KG.)	SERUM IRON (μG %)	UNBOUND IRON BINDING CAPACITY (μG %)	MEAN MAXIMAL Fe^{59} UPTAKE IN RED CELLS	HALF TIME OF PLASMA Fe^{59} DISAPPEARANCE (MINS.)	PLASMA IRON POOL (MG.)	PLASMA IRON TURNOVER RATE (MG./KG./DAY)	NO. OF TIMES/DAY PLASMA IRON POOL TURNED OVER	RED CELL IRON TURNOVER RATE (MG./KG./DAY)	RED CELL IRON RENEWED DAILY (%)	HALF TIME DISAPPEARANCE Cr^{51} RED CELLS	HEMOLYTIC INDEX†
32.0	54.2	37.5	91.6	80.9	350.0	84.9	75.0	2.37	0.59	13.4	0.50	1.15	-	9.2
31.0	50.2	27.7	77.8	83.0	261.0	83.6	71.0	2.47	0.63	15.1	0.53	1.59	-	13.3
31.7	40.4	29.0	69.4	98.0	250.0	74.1	74.5	2.14	0.53	13.5	0.39	1.12	-	-
34.0	33.5	27.8	61.4	106.0	100.0	85.0	84.0	3.17	0.42	11.8	0.36	0.81	-	-
34.0	46.2	33.1	79.1	100.0	125.0	87.0	75.0	2.69	0.64	13.3	0.56	1.28	-	-
32.0	38.6	23.8	62.4	60.0	200.0	76.0	96.5	1.80	0.24	10.4	0.18	0.61	-	-
32.0	37.8	27.1	66.4	100.0	150.0	79.0	114.0	2.90	0.34	8.5	0.28	0.81	-	11.9
34.0	42.9	31.5	74.8	57.5	200.0	92.6	58.0	1.88	0.42	17.3	0.39	0.91	-	11.9
32.0	54.1	41.5	78.2	165.0	100.0	84.4	148.0	6.53	0.53	6.0	0.45	0.84	-	7.2
32.0	40.4	28.8	69.5	91.0	200.0	68.8	94.0	2.61	0.52	14.2	0.36	0.95	-	-
32.4	43.8	30.1	71.3	94.1	193.6	81.5	89.0	2.85	0.49	12.4	0.40	1.01		10.7
	7.09	5.66	10.39	29.9	79.2	7.01	26.0	1.36	0.01	3.3	0.01	0.28		2.47
32.0	33.6	22.2	55.8	63.0	250.0	89.4	60.5	2.27	0.46	16.3	0.41	1.31	33	9.7
36.0	43.2	22.2	65.2	105.0	100.0	75.6	92.0	3.92	0.49	10.8	0.37	1.21	-	-
-	37.4	27.8	65.3	-	-	-	-	-	-	-	-	-	38	10.5
32.0	47.6	30.7	78.6	85.0	287.0	88.2	-	2.34	-	-	-	-	-	-
35.0	52.1	26.5	78.6	48.0	300.0	86.3	50.0	1.35	0.50	20.1	0.43	1.21	-	-
35.0	38.2	21.2	59.4	81.5	125.0	79.5	81.0	2.62	0.38	12.3	0.31	1.07	-	-
33.0	47.4	26.7	74.2	50.0	212.0	90.0	60.0	1.43	0.40	16.6	0.36	1.01	-	9.1
32.0	44.0	23.6	66.6	67.5	350.0	87.0	61.0	1.74	0.49	16.4	0.42	1.43	-	12.5
33.0	49.2	27.4	71.5	58.0	213.0	80.0	32.0	1.41	0.90	31.4	0.72	2.08	-	20.4
33.5	43.6	25.4	68.4	69.8	230.0	84.5	62.3	2.13	0.51	17.7	0.43	1.33		12.4
	6.14	3.21	3.02	19.49	85.7	5.37	19.0	0.86	0.17	6.76	0.01	0.36		4.64
30.2	51.8	16.6	68.4	-	-	-	-	-	-	-	-	-	26	22.0
31.3	57.5	20.0	77.5	13.8	300.0	47.5	41.0	0.37	1.47	18.7	0.70	3.17	19	24.0
33.9	51.6	17.8	69.4	-	-	-	-	-	-	-	-	-	25	17.7
29.5	36.6	10.8	47.4	64.2	185.0	38.7	30.0	2.44	0.93	17.8	0.36	1.21	16	56.8
31.4	41.7	18.9	60.6	49.0	282.0	46.8	58.0	1.55	0.40	17.2	0.19	0.81	17	21.1
31.8	40.4	24.2	64.6	-	-	-	-	-	-	-	-	-	38	9.4
34.0	45.2	10.6	55.8	-	-	-	-	-	-	-	-	-	11	48.0
36.1	47.0	18.7	65.8	88.0	218.0	31.7	56.0	3.31	0.70	16.6	0.22	0.96	-	13.9
33.0	54.5	17.6	68.1	67.9	150.0	32.6	-	2.34	-	-	-	-	-	-
34.0	50.1	14.5	64.5	-	-	-	-	-	-	-	-	-	13	15.4
34.0	50.4	20.8	72.8	17.2	150.0	66.0	34.0	0.60	0.26	28.8	0.17	0.59	-	10.0
32.0	48.3	13.0	61.4	62.0	162.0	37.0	80.0	1.63	0.37	12.5	0.14	0.65	-	19.2
32.0	66.4	24.9	91.2	50.5	222.0	56.0	79.0	1.96	0.39	12.6	0.22	0.65	-	-
30.0	59.3	18.1	77.1	42.0	188.0	42.0	83.0	0.98	0.29	15.2	0.12	0.43	-	-
33.0	48.1	13.1	61.3	87.0	50.0	27.0	138.0	2.25	0.82	7.2	0.82	0.49	-	-
32.4	49.9	17.3	67.1	54.0	191.0	42.5	66.5	1.74	0.62	16.3	0.32	0.99		23.4
	7.64	4.32	10.25	25.24	71.5	11.92	33.4	0.91	0.38	5.89	0.24	0.84		15.1
	1.0	0.05	0.5	0.1	0.4	0.4	0.05	0.3	0.05	0.7	0.7	0.1		0.5
	0.1	0.001	0.4	0.01	1.0	0.001	0.2	0.05	0.1	0.3	0.8	0.07		0.1
	0.05	0.001	0.8	0.2	0.4	0.001	0.8	0.4	0.7	0.6	0.4	0.4		0.2

†Hemolytic index = $\frac{\text{fecal urobilinogen Gm./day} \times 100}{\text{total circulating Hb.}}$.

patient with renal disease. There are 40 values. Those patients not included in the other parts of this study were ones who were under observation but had been considered unsuitable for various reasons, most usually the absence of azotemia. A definite negative correlation is seen, as the B.U.N. rises the hemoglobin falls. Statistically p is less than 0.001 which is highly significant. For each 8 mg. per cent rise in B.U.N. the hemoglobin falls approximately 1 Gm. per cent over the range of values studied.

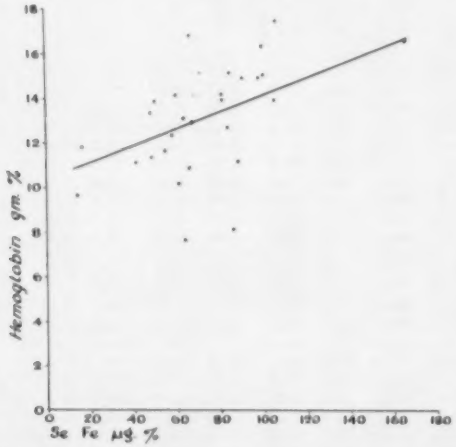

Fig. 2.—Relationship between hemoglobin and serum iron ($r = 0.517$, $p < 0.01$).

The fall in hemoglobin in Groups 2 and 3 is paralleled by significant falls in both red blood cell count and hematocrit. The anemia is, however, normocytic and normochromic, the corpuscular constants remaining unchanged. The plasma volume likewise remains unchanged and actually increases in Group 3. This is true whether calculated as total amount or milliliters per kilogram and is, therefore, not due to a fall in body weight. Total blood volume remains unchanged. Aspiration bone marrows were done on all patients in Groups 2 and 3. No gross morphologic changes were seen.* Quantitative differential counts in many cases were likewise noncontributory; however, no detailed study of bone marrow smears was attempted as this has already been carefully done.[17]

Although the value for total iron binding capacity in the serum remains constant, the serum iron tends to fall as renal failure becomes more marked. The difference between Groups 1 and 3 is highly significant ($p < 0.01$). Considerable variation exists between different patients, however, in individual values and for this reason owing to the small size of the groups, Groups 1 and 2 do not differ statistically at the 5 per cent level although the mean of Group

*We are indebted to Dr. Lorne Shapiro, Consultant Hematologist, for reading the marrow slides.

2 is considerably less than that of Group 1. Nevertheless the trend seems quite definite. There seemed to be some correlation between serum iron and hemoglobin ($p < 0.01$) as shown in Fig. 2 but none between serum iron and B.U.N.

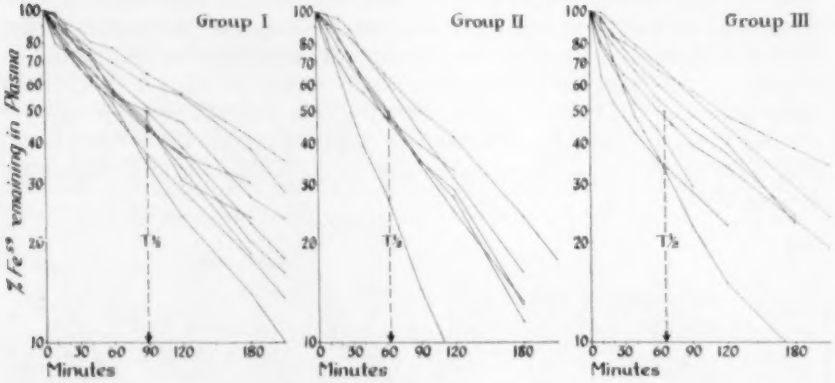

Fig. 3.—Rate of disappearance of injected Fe^{59}.

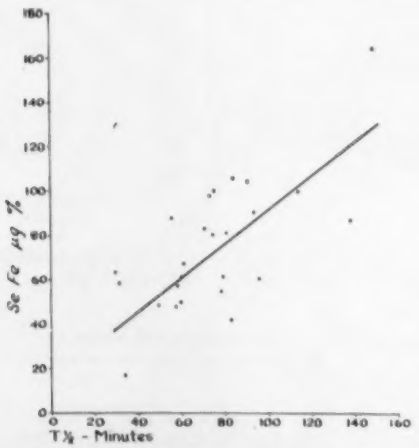

Fig. 4.—Relationship between serum iron and plasma iron disappearance rate ($r = 0.710$, $p < 0.001$).

Following injection of tracer iron, the rate of removal from the plasma was followed in the 3 groups. The time for one-half of the injected iron to have disappeared was less for both Groups 2 and 3 as compared with the controls. (Figs. 3 and 4.) Patient R. A. in Group 3 was an exception inasmuch as his plasma clearance time was markedly prolonged. He was severely uremic at the time of the study and this may have in some way affected his plasma clearance. If he is exceluded, then the difference between the means of both Groups 2 and 3 from the controls are significant ($p < 0.05$ and 0.02, respectively).

Following removal during the first day, the radioiron becomes available to the maturing red cells, incorporated into hemoglobin, and appears in the circulating erythrocytes. A major difference is apparent in those patients with less than 12 Gm. per cent hemoglobin, all contained in Group 3, in comparison to the rest of the patients with renal disease and the control group. (Fig. 5.) Without exception, all in Group 3 incorporated less than would be expected of the iron, with a mean utilization value of 42.5 per cent. Both other groups utilized an average of 81.5 per cent and 84.5 per cent with no overlap in the individual figures between Group 3 and the other two groups. Fig. 6 shows that as the hemoglobin falls so the utilization of radioiron decreases.

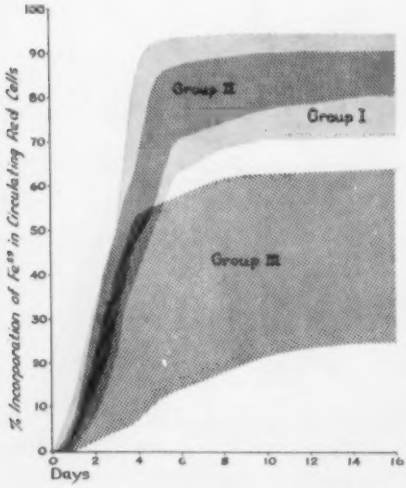

Fig. 5.

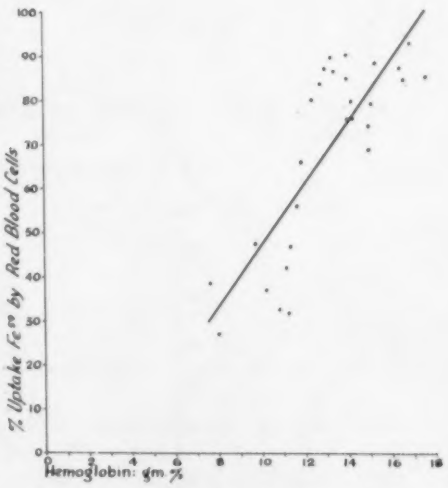

Fig. 6.

Fig. 5.—Incorporation of Fe^{59} into circulating erythrocytes.

Fig. 6.—Relationship between hemoglobin and per cent incorporation of Fe^{59} into circulating erythrocytes ($r = 0.820$, $p < 0.001$).

The above changes in iron metabolism are illustrated by patient O. F. who is the only one to appear in both Groups 2 and 3. Throughout the whole period of observation extending over 14 months this patient's general health was excellent and he was asymptomatic. The almost accidental discovery of an abnormal urine led to the diagnosis of acute glomerulonephritis which slowly healed over a period of 4 months. This diagnosis was confirmed by renal biopsy. Two studies of iron metabolism were performed at intervals of 3 months. (Fig. 7.) The first one reveals the characteristic changes present in the patients in Group 3. The second shows the shift of these values to the normal range.

Sixteen normal subjects have had erythrocyte survival times estimated in this laboratory using the chromium^{51} method. The results agree closely with

those reported by others with 50 per cent of the labeled cells disappearing from 25 to 43 days with a mean of 29.8 days. Ten patients in Group 2 and in Group 3 had survival times done. Unfortunately, it was not possible to do these simultaneously with the radioiron studies, as apparatus for discriminating between the two isotopes was not available when most of these data were being collected. The estimates of survival time were, therefore, obtained several months before or after the radioiron studies and because of the long interval the only patients in whom survival times were estimated were those with a stable hematologic and renal state. It is possible that some of the other patients who were not studied would also have shown abnormalities in erythrocyte life span and the number of patients proved to have shortened survival times probably represents only a portion of the total group.

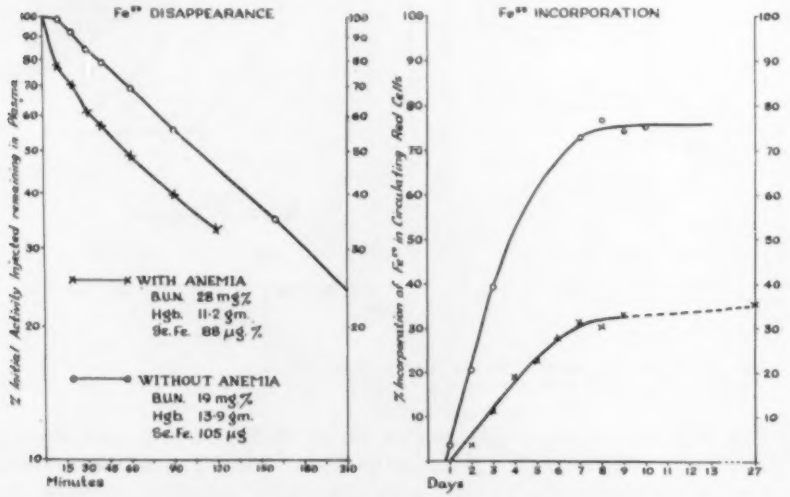

Fig. 7.—Changes in iron metabolism in Patient O. F.

Of the 10 patients studied, 5 were found in whom red cell life span was significantly shortened; they were W. L., J. L., M. D., L. S., and C. W. All were in Group 3. Patient W. L. with severe chronic pyelonephritis has been under continuous observation for 18 months. Three separate survival times were carried out. On the first occasion (the topmost line in Fig. 8), following labeling of his own erythrocytes the activity in the circulating blood instead of falling remained almost stationary over a period of 8 days. This was accompanied by a fall in hematocrit from 25 per cent to 18 per cent. There was no evidence of blood loss and repeated stools were negative for blood. These findings were interpreted as evidence of a decreased output of new red cells by the marrow but with continued destruction of aged cells, comparable in some ways to the crises described in other hemolytic states, notably congenital spherocytosis.[18] When the hematocrit had fallen to 18 per cent the patient

was transfused with 500 ml. whole blood and 800 ml. packed cells. Subsequently his erythrocyte survival time was estimated and found to be markedly shortened. The patient's cells were then injected into a normal recipient after labeling with radio-chromium where a normal survival time was found. Normal compatible cells transfused into the patient showed a markedly shortened life span.

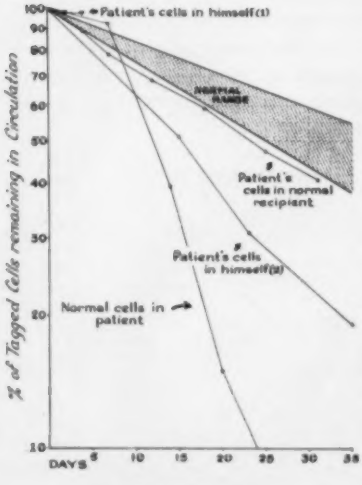

Fig. 8.

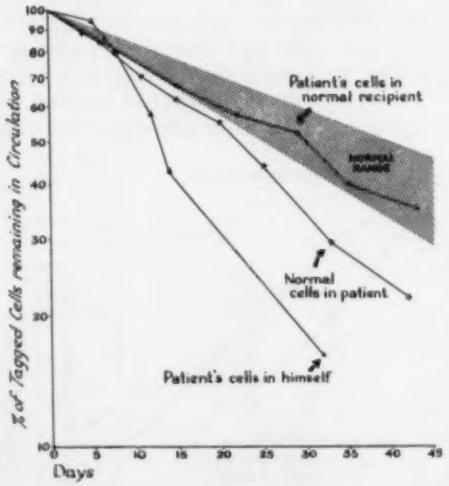

Fig. 9.

Fig. 8.—Erythrocyte survival time in Patient W. L.
Fig. 9.—Cr^{51} survival data in Patient J. L.

Fig. 9 shows an essentially similar set of findings. J. L. had chronic pyelonephritis and was clinically similar to W. L. The degree of renal failure showed no appreciable change during an observation period of one year. In October, 1956, his own erythrocytes in himself had a shortened life span. The same cells in a normal compatible recipient had a normal life span. In August, 1957, there had been no change in his condition. At this time normal cells transfused into the patient showed a shortened life span.

Patient M. D., Fig. 10, when having his erythrocyte survival time estimated was found to have developed a reticulocytosis which varied from 1 to 4.6 per cent. The patient's red cells were transfused into a normal recipient at a time when the reticulocytosis was at peak values and his own red cell survival time was shortened. In the normal subject these cells had a normal survival time. At this time his clinical condition was excellent with compensated renal failure which had shown no change over a 6-month period. After allowing an interval of 2 months to eliminate the residual radioactivity from his own cells, he was transfused with normal red cells. It was surprising to find the survival time was now normal. The reticulocyte count had also

decreased. Following this, repeat labeling of his own cells did indeed now show a survival falling with the normal range. The hemolytic process here was apparently a transitory one.

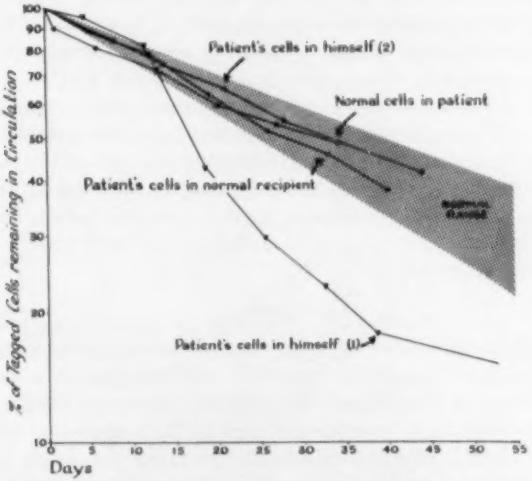
% of Tagged Cells remaining in Circulation
Patient's cells in himself (2)
Normal cells in patient
Patient's cells in normal recipient
Patient's cells in himself (1)
Days

Fig. 10.—Cr^{51} survival data in Patient M. D. For explanation, see text.

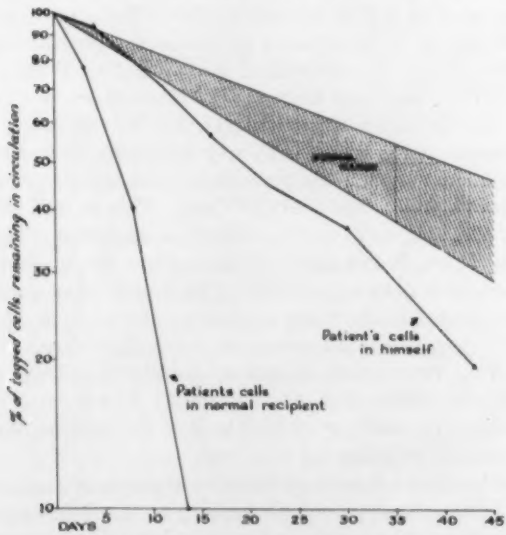

Fig. 11.—Cr^{51} survival data in Patient L. S.

Patient L. S., Fig. 11, did not differ clinically except that his renal failure was not as severe as the other 4 patients. The survival time of his erythrocytes

in himself was shortened but surprisingly they were also shortened in a normal recipient. Group compatibility was rechecked in this instance as was the recipient's blood after an interval for the development of antibodies by the Coombs' technique, but no abnormalities were uncovered. Unfortunately it was impossible to recheck those results as the patient moved to another district.

Although evidence of a hemolytic process was essentially derived from the cell survival studies in these patients, it was noted that the reticulocyte counts tended to be above 2 per cent and therefore higher than most other patients in Group 3. Furthermore the excretion of urobilinogen in the feces was high relative to the red cell mass. Although the values for Group 3 as a whole are not statistically different from Groups 1 or 2, yet if the hemolytic indices of the 5 patients with accelerated erythrocyte destruction are compared with the other patients in Groups 1 and 2 then the difference between the means is significant ($p < 0.05$).

DISCUSSION

Anemia is not an invariable or even common accompaniment of renal disease because in most instances the condition will be present without having progressed to the extent of sufficient renal failure to give azotemia. Although there is no direct evidence to incriminate urea as the responsible agent, nevertheless the level of this retained metabolite provides the single most useful guide as to when anemia may be expected, and of approximately what severity. Even so it is clear from Fig. 1 that the variation in hemoglobin between different patients at any level of B.U.N. is considerable. The reserve of an individual's bone marrow may be of importance as well as the presence of more than one mechanism for anemia. In addition, if the level of renal failure is changing then the hemoglobin may not keep pace. There is often a latent period of several weeks during which time with the B.U.N. rising the hemoglobin will show little change. After the B.U.N. may have stabilized or even started to fall then the hemoglobin may fall profoundly and not rise again until the B.U.N. has been normal for a considerable time. This is well illustrated by Fig. 12 of patient M. d'A. with acute glomerular nephritis. The maximum fall in hemoglobin is not found until 20 days after the highest B.U.N. A similar sequence has been seen repeatedly. The initial delay probably represents the time required for the normal random destruction of circulating erythrocytes to have progressed far enough to lower the red cell mass appreciably. During this time the marrow is depressed and the delivery of new cells is less than the rate of destruction of aged ones. The reverse occurs after the B.U.N. has fallen, the marrow requiring time to increase the output of new cells to restore the circulating red cell level.

From what has been said it is clear that the presence of anemia in a given patient with renal disease is of no aid whatsoever in the differential diagnosis of the underlying disease. If we exclude from consideration increase in plasma volume without decrease in red cell mass, then the presence or absence of anemia is related solely to renal failure which can be produced by any one of innumerable different conditions.

Likewise it is worth emphasizing the pitfalls of using the presence of anemia for prognosis. Unless very severe, the anemia of itself carries no special hazard and the prognosis is, therefore, that of the primary condition, namely, the renal disease. If this is acute, for example, acute glomerular nephritis, because of the favorable tendency for healing or at least remission of this stage of the disease the prognosis can be favorable, even if anemia is marked. In chronic disease, glomerular nephritis and pyelonephritis may be taken as examples. Similar levels of blood urea nitrogen and hemoglobin may be seen when the patient first comes under observation, yet the patient with glomerular nephritis is likely to die much sooner than that with pyelonephritis. This is the natural history of the disease irrespective of the degree of anemia.

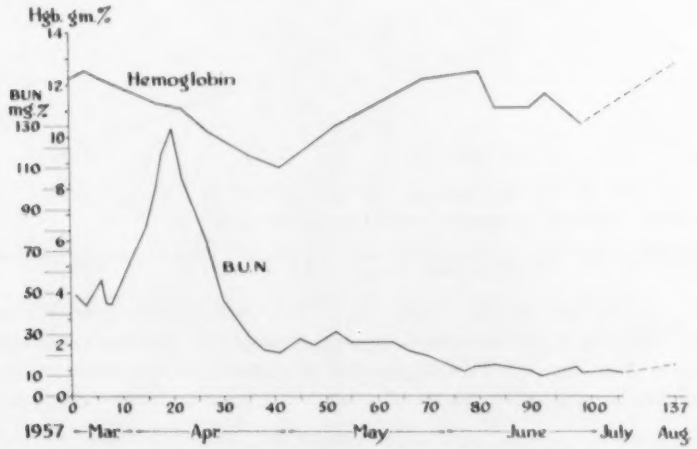

Fig. 12.—Patient M. d'A. showing relationship of B.U.N. and hemoglobin during acute glomerular nephritis.

From the data presented there is evidence that two different processes may lead to anemia in the presence of renal disease. The first is reflected in the radioiron data, the second in the radio-chromium survival times. It is clear that both these mechanisms need not be present in the same patient. Although the cell survival data are small there are at least 3 patients in Group 3 (N. L., F. H., and A. D.) with marked anemia who had normal erythrocyte life spans. On the other hand all in Group 3 had profound disturbances of iron metabolism most clearly demonstrated in the depression of marrow utilization of the injected tracer iron. Similarly although this parameter was not disturbed in Group 2 patients, yet there were definite indications (e.g., the plasma iron clearance rate) that iron metabolism was abnormal in this group as well. These findings make it likely that every patient who has anemia secondary to renal disease will show evidence of abnormal iron metabolism. A hemolytic process may or may not be present, but if it is, it will be probably superimposed on the changes in iron metabolism which have been described.

The pathophysiologic interpretation of the radioiron data is difficult. There is a fall in serum iron which reflects a decrease in total intravascular circulating iron. Any iron introduced into the circulation is removed more rapidly than normal. This is reflected in the number of times a day the plasma

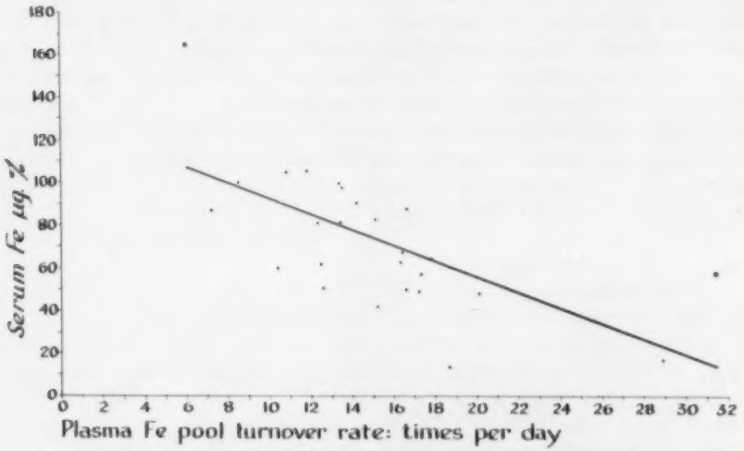

Fig. 13.—Relationship between the serum iron and the number of times the plasma iron pool is turned over daily ($r = 0.654$, $p < 0.001$).

iron pool is being replenished. (Fig. 13.) Yet, evidence for iron deficiency is lacking. Patient G. D., who has not been included in the previous data, is of interest in this respect. This 38-year-old man was admitted with profound anemia (Table II) and active glomerular nephritis with azotemia. The anemia

TABLE II. SHOWING RESPONSE OF PATIENT G. D. TO IRON THERAPY

DATE	HB. (GM. %)	R.B.C. CELLS/ CU. MM. × 10^6	HMCT. (%)	RETICS. (%)	B.U.N. (MG. %)	SERUM FE (μG %)	I.B.C. (μG %)	THERAPY
Dec. 5, 1956	6.0	2.5	21	1.7	28	12	437	Oral ferrous gluconate 0.33 Gm. 3 times daily
Dec. 10, 1956	6.0	3.0	24	8.0				
Dec. 15, 1956	7.2	2.9	25	6.6				Intramuscular iron started
Jan. 8, 1957	8.4	2.8	28	0.5	54			Intramuscular iron stopped. Total 1,300 mg.
Feb. 4, 1957	8.6	2.8	27	0.6	101	45	230	Ferrous gluconate stopped. Total 61 Gm.
Feb. 19, 1957	7.0	2.5	21	2.2	124			

was clearly iron deficient. The plasma iron clearance was very rapid and the per cent utilization was within the normal range, yet less than expected for pure iron deficiency. (Fig. 14.) He received a total of 1,300 mg. elemental

iron intramuscularly and 7.0 Gm. by mouth. It can be seen from the reticulocyte figures that oral iron was well absorbed. The hemoglobin rose to 8.6 Gm. per cent only and subsequently fell as renal failure became more severe. All evidence of iron deficiency disappeared. He was now left solely with anemia secondary to renal disease. The still markedly lowered serum iron is notable.

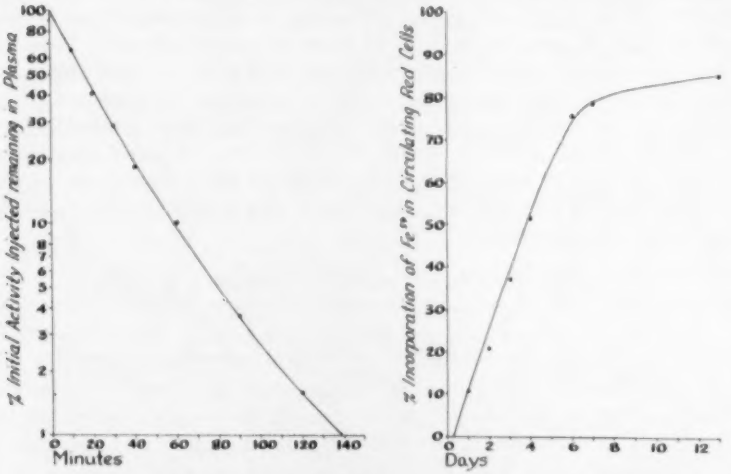

Fig. 14.—Patient G. D. showing disappearance and incorporation of Fe^{59}.

It was noted that in several instances (Fig. 3, Group 3), the slope of the plasma iron disappearance tended to depart from linearity and become curved. A similar phenomena has been noted previously and suggests exchange with other labile iron systems situated extravascularly.[19]

The evidence suggests a rapid storage process whereby iron is being removed from the plasma pool. A similar process has been described previously in rats with turpentine abscesses.[20] It was of interest to determine the site of this storage and to see if the iron which in the Group 3 patients was not accounted for in the peripheral blood could be found elsewhere.

The results in 2 patients are shown in Table III. Both had been studied earlier and are included in Group 3. J. P. was uremic but his general condition was good; he was conscious and able to read the newspaper. There had been no blood loss. He died suddenly from a cerebral hemorrhage. R. A. was in excellent condition, up and around the ward. He suddenly went into pulmonary edema and died. No precipitating cause could be discovered at autopsy. Both died about 2 months after the initial radioiron studies. In each case this was the only isotope administered. The total organs were weighed separately. Small aliquots 3 to 4 Gm. in size were lightly dried with gauze and weighed on a torsion balance. They were wet ashed with concentrated nitric acid until the solution was clear, made up to 10 ml. volumes and a 5 ml. aliquot was

counted in the same way as the initial radioiron samples. Total activity present in each organ was found by multiplying by the autopsy weight. No allowance was made for contained blood. The activity of the red cell mass was assumed to remain constant from the time of the last determination. As a plateau had been reached the error was probably small. It can be seen from the figures that the radioactivity of the various organs is minimal with the exception of the liver and spleen. Together these accounted for 83 per cent in R. A. and 96 per cent in J. P. of the total activity found. Total recovery was 102 per cent in J. P. but only 58 per cent in R. A. The reason for this is not known, as loss outside the body can probably be excluded. The urine following the original injection of radioiron had been counted in this case, as well as several others in Group 3 and the activity was negligible in each instance. It may be noted here that proteinuria in all these patients was small, usually below 2 Gm. per day, and loss of iron bound to protein would not be expected to be significant.

TABLE III. AUTOPSY TISSUE COUNTS FOR Fe^{59} IN PATIENTS J. P. AND R. A.

NAME	ORGAN	ACTIVITY COUNTS/SEC. PER GM. WET WEIGHT	TOTAL ORGAN ACTIVITY (COUNTS/SEC.)	PER CENT OF RECOVERED Fe^{59} TISSUE AND BLOOD	TOTAL PER CENT RECOVERY OF INJECTED Fe^{59}
J. P.	Heart	0.28	139	> 1.0	102
	Lungs	1.11	700	1.7	
	Liver	12.39	22,300	52.6	
	Spleen	51.90	9,070	21.4	
	Kidney	1.33	225	> 1.0	
	B. marrow	1.10			
	Blood		9,860	23.3	
R. A.	Heart	0.00	0	0.0	58
	Lungs	0.46	647	5.8	
	Liver	2.86	4,620	41.5	
	Spleen	3.41	1,025	9.2	
	Kidney	3.00	539	4.9	
	B. marrow	0.30			
	Blood		4,300	38.6	

The results from these 2 patients strongly suggest that iron is being preferentially retained in the liver and spleen, of which the liver is quantitatively the most important. It remains to be considered whether this is a primary defect in iron metabolism or whether secondary to reduced marrow activity. The latter seems most likely in the Group 3 patients where the utilization of iron by the marrow is depressed yet the red cells are not iron deficient. A similar process may be taking place in the Group 2 patients where there is reduced erythroblastic activity but to a lesser degree so that utilization of iron remains grossly normal yet the "stockpiling" of iron which is in excess of the marrow's ability to use is starting to take place. Similar findings to these have been reported in patients anemic due to rheumatoid arthritis[19] and infection.[21]

The hemolytic process in 5 patients was shown not to be due to any structural intrinsic defect in the red cell in 3. No antibodies could be detected although looked for by the Coombs' technique and also in saline, albumin, and

in the cold in each instance. Recent evidence demonstrating abnormalities of intermediary red cell metabolism in uremic patients[22] suggests that hemolysis, when present, is due to increased susceptibility of the red cell envelope to disruptive influences which, although experienced continuously by normal erythrocytes, leads to premature death of uremic cells. This defect might be correctible with a change in environment as, for example, after transfusion into a normal recipient. However, an attempt to demonstrate alteration in the mechanical fragility of patient W. L.'s cells when suspended in his own plasma as compared with normal plasma was unsuccessful. Similarly there was no effect when normal cells were suspended in W. L.'s plasma. Osmotic fragilities were measured and found to be normal in all those with shortened erythrocyte life spans.

The insidious nature of the hemolytic process in these patients has been noted by others and is being described in a variety of other disease states. Reticulocyte counts although at times over 2 per cent never exceeded 6 per cent and were rarely as high as that. Fecal urobilinogen tended to be moderately increased when related to red cell mass. Serum bilirubin was invariably normal.

Any discussion of anemia in renal disease would be incomplete without referring to treatment. In the uncomplicated anemia under review in this paper, there is clearly no need for iron. Its limitation may well be seen in Patient G. D. Likewise there is no evidence of deficiency of vitamin B_{12}, folic acid, or other known maturation factors. Cobalt will undoubtedly raise the hemoglobin of some patients but we have had no experience with it. It is common practice, however, to transfuse patients of the type described in this paper. We believe that in most instances this is not indicated. Naturally replacement of blood loss following hemorrhage in the uremic state is an entirely different matter, but in the slowly developing anemia as seen in our patients acclimatization to a reduced red cell mass appears to take place quickly. Blood volume is well maintained. Blood transfusion apart from the inherent risk of viral hepatitis and incompatibility will expand blood volume above normal thereby increasing the danger of pulmonary edema by overloading the heart. Having elevated the hemoglobin it will usually fall to the pretransfusion level within the life of the transfused cells or faster if there is a hemolytic component. Mere elevation of hemoglobin probably does not improve renal function and there is evidence of acclimatization of filtration rate and plasma flow to marked differences in hematocrit.[23, 24] The circumstances in Patient W. L. are unusual, but if relative marrow failure can develop then the data suggest transfusion may establish or permit a new level of marrow function which will be maintained thereafter.

SUMMARY

The anemia associated with renal failure has been investigated from the standpoint of natural history, radioiron metabolism, and erythrocyte survival. A marked disturbance in iron metabolism probably secondary to marrow

dysfunction is apparent with the development of anemia. In some instances a hemolytic component appears. The severity of the anemia correlates fairly well with the degree of renal failure.

We particularly wish to thank Dr. G. W. Halpenny without whose support and encouragement this study would not have been possible and, also, Dr. A. H. Neufeld for advice and facilities provided and Miss V. Davidson for secretarial assistance.

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THE COAGULATION OF BLOOD BY RUSSELL'S VIPER VENOM

A Reaction Between Russell's Viper Venom and Beef Serum Factors

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THE ability of Russell's viper venom (RVV) to promote rapid clotting of recalcified plasma[1] led to the concept that RVV was a thromoplastin similar to brain thromboplastin. Subsequently, however, major points of difference between the tissue thromboplastins and RVV were observed, notably that plasmas from patients receiving Dicumarol frequently showed clotting times with RVV much shorter than with brain or lung thromboplastin,[2] and that either platelets or certain lipids were required for maximum activity of RVV, whereas brain and lung thromboplastins were not as dependent on these factors.[3, 4]

In some respects RVV-lipid mixtures resemble the thromboplastic activity which normally develops in mixtures of $Al(OH)_3$-treated plasma, serum, and platelets.[5] In this comparison it appears that the addition of RVV (and phospholipid) to recalcified citrated plasma shortens the clotting time by providing an activity similar to some product which normally arises in the generation of plasma thromboplastin only after some delay. Biggs, Douglas, and Macfarlane[6] showed that very short clotting times can be obtained by the addition of aliquots of an incubation mixture containing RVV, serum, platelets, and factor V to normal plasma or to fibrinogen-prothrombin mixtures.

Factors which are presently recognized as being necessary for the maximum activity of RVV in shortening the coagulation time of recalcified plasma include calcium, a lipid factor[3, 7] or platelets,[3] factor V (proaccelerin, labile factor),[6, 7] and perhaps Stuart factor,[8] but the reactions in which RVV is involved have not been characterized.

We will describe a reaction between RVV and factors present in beef serum and show that the product of this reaction is active in the coagulation of plasma. We will present evidence that phospholipid is not required for the reaction between the venom and the beef serum factors but is required in some subsequent reaction.

METHODS AND MATERIALS

We have used two-stage systems for the study of the coagulant activity of RVV. Dilute solutions of the venom were incubated with dilutions of beef serum and solutions

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of other clotting factors. Aliquots of the incubation mixtures were removed at intervals and added to samples of plasma or fibrinogen solution, and the clotting times were noted.

Russell's viper venom (RVV) is the commercial product Stypven.*

Commercial† sterile calf serum was separated in 1 to 2 ml. aliquots and kept frozen until just before use. It was devoid of prothrombin (as measured by the two-stage technique) and had no thrombic or thromboplastic activity. It corrected the prolonged one-stage prothrombin time of patients in the early phase of Dicumarol therapy and served adequately as a source of accelerators in the two-stage prothrombin reaction.

All solutions except fibrinogen were made in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4.

A 2 per cent solution of Armour's bovine fraction I in imidazole buffer was used for fibrinogen.[9]

Phosphatidyl ethanolamine (PE) was fraction 5 of crude brain cephalin prepared according to Folch.[10] It was suspended in Tris buffer by shaking 10 minutes on a mechanical shaker.

$Ca(Cl)_2$, 0.02 M, was prepared in Tris buffer.

Substrate plasma was obtained from normal volunteers. Using siliconed syringes, 10 to 20 ml. samples of blood were drawn and placed in siliconed tubes containing 0.1 volume of 0.11 M sodium citrate anticoagulant. The whole blood was centrifuged in the cold and the plasma separated. The plasma was then centrifuged at 105,000 g for 30 to 45 minutes in a Spinco Model L ultracentrifuge. The lipid layer which accumulated at the surface was carefully pipetted off and discarded. The "lipid-poor" plasma which remained was then carefully decanted from any sediment and used promptly. The removal of lipid in this manner assured that the effect of added purified phospholipid would be more uniform with different plasma samples. Specimens of plasma prepared without the precautions described above varied widely in RVV-clotting times and were not used. Although plasmas prepared carefully gave more uniform results, slight differences in clotting times were noted. Only one specimen of plasma was used for all of the data in any one figure or table. Data in different tables or figures should not be compared.

Purified prothrombin was prepared from human plasma according to Owren.[11] Prothrombin activity was assayed by the two-stage technique of Warner, Brinkhous, and Smith[12] as modified by Ware and Seegers[9] and was found to be approximately 80 units per milliliter. The prothrombin so obtained was probably contaminated by factor VII (stable factor), but was inactive in the two-stage procedure in the absence of dilute beef serum.

Incubation mixtures were kept at 37° C. in a constant temperature water-bath and plasma or fibrinogen for clotting was prewarmed to 37° C. The moment at which a definite web of granules appeared was the end point chosen for determining the clotting times of plasma. The first appearance of definite fibrin threads was the end point for fibrinogen clotting times.

RESULTS

Solutions of RVV in concentrations of the order of 0.001 mg. per milliliter possess very weak coagulant activity. When such dilute solutions of RVV are incubated with beef serum, calcium, and PE, however, there appears in the incubation mixture a potent coagulation accelerating activity which increases with time and reaches a maximum within a few minutes (Table I). The omission of the beef serum results in a failure of the mixture to develop such activity. Beef serum dialyzed against 0.85 per cent NaCl retains its effectiveness in this system. Beef serum which has been heated to 56° C. for 30 minutes shows greatly reduced activity.

*Burroughs, Wellcome & Company, Tuckahoe, N. Y. (Dried venom obtained from the Ross Allen Reptile Institute, Silver Springs, Fla., was found to be equivalent in our system.)

†Cappel Laboratories, West Chester, Pa.

TABLE I. CLOTTING TIME IN SECONDS OF 0.1 ML. OF LIPID-POOR PLASMA UPON SIMULTANEOUS ADDITION OF 0.1 ML. OF INCUBATION MIXTURE AND 0.1 ML. OF 0.02 M $Ca(Cl)_2$

INCUBATION MITXURE (37° C.)	INCUBATION TIME (MIN.)									
	1	2	3	4	5	6	7	8	9	12
Complete*	22	16½	13	12½	11½	11½	11½	–	11½	–
Buffer replaces RVV	85	–	82½	–	–	78	–	77½	–	–
Buffer replaces beef serum	64	–	–	50	–	54½	–	55½	51	–
Buffer replaces PE	100	–	–	75	–	–	67½	–	–	67
With heated beef serum†	36½	–	35	–	34	–	36½	–	33	–
With dialyzed beef serum‡	20½	–	15	–	13½	–	13	–	–	–

*Complete incubation mixture includes equal volumes of RVV (0.004 mg. per milliliter); PE (0.2 mg. per milliliter); beef serum (1:10 dilution); and $Ca(Cl)_2$ (0.02 M).

†Normal beef serum replaced by beef serum heated at 56° C. for 30 minutes.

‡Normal beef serum replaced by beef serum after dialysis against 0.85 per cent NaCl for 18 hours at 4° C.

Some thrombin appears in the complete incubation mixture, presumably from the conversion of traces of prothrombin (not measurable by the two-stage technique) which remain in the beef serum.* When a 2 per cent solution of fibrinogen is used as clotting substrate instead of recalcified plasma, clotting times in the range of 120 to 150 seconds are observed. One-tenth milliliter of a solution of commercial thrombin† containing approximately 0.1 unit of thrombin per milliliter clots the same volume of the fibrinogen solution used here in much less than 120 seconds. If this amount of thrombin is added to substrate plasma simultaneously with an aliquot of the complete RVV incubation mixture and calcium, or if 4 times as much thrombin is added to the incubation mixture, the clotting times of the substrate plasma are not significantly changed.

TABLE II. CLOTTING TIME IN SECONDS OF 0.1 ML. OF LIPID-POOR PLASMA UPON THE SIMULTANEOUS ADDITION OF 0.1 ML. OF INCUBATION MIXTURE AND 0.1 ML. OF 0.02 M $Ca(Cl)_2$

(0.1 ml. of PE or of Tris buffer is added to plasma just prior to addition of incubation mixture and calcium)

INCUBATION MIXTURE	ADDED TO PLASMA 0.1 ML. OF:	INCUBATION TIME (MIN.)						
		1	2	3	4	5	6	7
Complete*	Tris buffer	18½	14½	12½	11	11	–	10½
Complete	0.05 mg./ml. PE	19½	17½	16	13	14	–	13
Buffer replaces PE	0.05 mg./ml. PE	21	15	15½	13½	–	13½	13½
Buffer replaces PE	Tris buffer	55	–	43½	39	37	–	38½

*Complete incubation mixture as in Table I.

Although the omission of PE from the incubation mixture appears to prevent the development of activity (Table I), it was demonstrated that PE is not required for the reaction between the RVV and the beef-serum factor(s). When PE was omitted from the incubation mixture, activity developed and could be demonstrated, provided the phospholipid was added to the plasma to be clotted (Table II). The addition of PE to the plasma just prior to, rather than with, the incubation mixture, as in the previous experiments, has the effect of increasing somewhat the clotting time of the plasma; however, the

*The amount of thrombin developed in the incubation mixture varied with different lots of beef serum employed.

†Thrombin, Topical (Bovine origin), Parke, Davis & Company, Detroit, Mich.

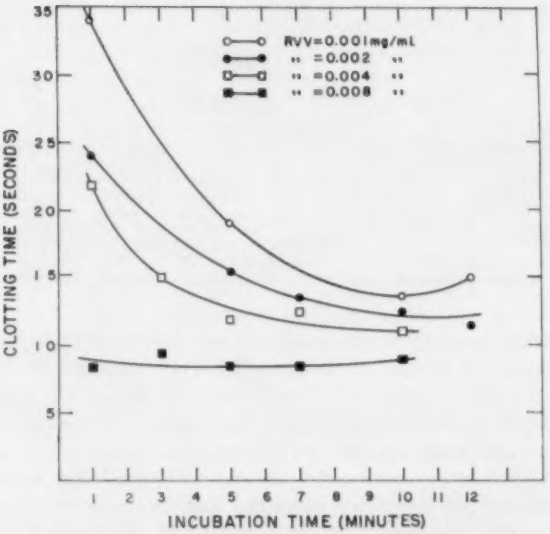

Fig. 1.—Incubation mixture contains equal volumes of RVV solution, dilute beef serum, and $Ca(Cl)_2$. At intervals, 0.1 ml. of incubation mixture and 0.1 ml. of $Ca(Cl)_2$, 0.02 M, were added simultaneously to 0.1 ml. of lipid-poor plasma to which 0.1 ml. of PE (0.05 mg./ml.) had been added 15 seconds previously. The clotting time of the plasma is recorded in seconds. The concentration of the RVV solution added to the incubation mixtures was varied as indicated. The dilution of beef serum added to each incubation mixture was 1:5.

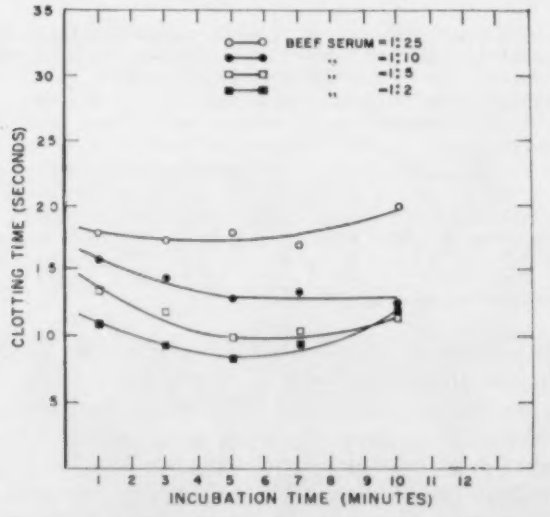

Fig. 2.—As for Fig. 1, except that the concentration of the RVV solution added to each incubation mixture was 0.004 mg. per milliliter. The dilution of beef serum added to the mixtures was varied as indicated.

development of coagulant activity during the RVV-beef serum incubation appears to be the same in either case. The beef serum was required in the incubation mixture with the RVV (Table III). It was without effect if added at a later stage.

TABLE III. AS IN TABLE II, EXCEPT THAT 0.025 ML. OF 1:10 DILUTION OF BEEF SERUM OR OF TRIS BUFFER ADDED TO PLASMA JUST PRIOR TO ADDITION OF INCUBATION MIXTURE AND CALCIUM

INCUBATION MIXTURE	ADDED TO PLASMA 0.025 ML. OF:	INCUBATION TIME (MIN.)							
		1	2	3	4	5	6	7	8
Complete*	Tris buffer	17	13	11	11	11	11	11	–
Complete	Dilute beef serum	–	16	14	–	12½	–	12½	–
Buffer replaces beef serum	Tris buffer	35	35½	–	36	–	37	–	37
Buffer replaces beef serum	Dilute beef serum	39	–	40	–	40½	40½	–	42½

*Complete incubation mixture as in Table I.

In order to determine whether or not the product of the incubation mixture could convert prothrombin to thrombin without the participation of other factors present in the plasma, a purified prothrombin preparation was added to the incubation mixture. Under these conditions, thrombin was produced (Table IV). Virtually no thrombin production was observed in the absence of either beef serum or PE.

TABLE IV. CLOTTING TIME IN SECONDS OF 0.1 ML. OF FIBRINOGEN SOLUTION AT 37° C. UPON ADDITION OF 0.3 ML. OF INCUBATION MIXTURE CONTAINING PURIFIED PROTHROMBIN

INCUBATION MIXTURE AT ROOM TEMPERATURE	INCUBATION TIME (MIN.)												
	1	2	3	4	5	6	7	8	9	10	20	25	30
Complete*	>120	–	31	27½	–	22	–	19	–	19	–	–	–
Buffer replaces RVV	>300	–	>300	–	–	–	>300	>300	–	–	115	–	75
Buffer replaces beef serum	>300	–	290	–	–	246	–	–	–	–	–	–	155
Buffer replaces PE	>300	–	>180	–	–	–	95	–	–	83	–	65	50

*Final concentration in complete incubation mixture of: RVV = 0.0025 mg. per milliliter; PE = 0.025 mg. per milliliter; beef serum = 1:1,000 dilution; $Ca(Cl)_2$ = 0.0025 M; prothrombin = approximately 10 units per milliliter.

The nature of the RVV-beef serum reaction was investigated by varying the concentrations of the two components independently. Increasing the concentration of the RVV while keeping the concentration of beef serum constant (Fig. 1) decreases the incubation time required for development of maximum activity and somewhat increases activity; decreasing the concentration of beef serum with constant venom concentration (Fig. 2) decreases the maximum activity developed but does not appreciably alter the time necessary for the development of this maximal activity. The omission of calcium from the incubation mixture significantly impairs but does not prevent the development of activity.

DISCUSSION

We have demonstrated that RVV participates in a time-consuming reaction with factors present in beef serum to form a product which, in the presence of phosphatidyl ethanolamine, will bring about rapid clotting of plasma and conversion of a partially purified prothrombin. Even very small quantities of the venom can bring about production of a potent coagulant activity, given sufficient incubation time.

The data shown in Figs. 1 and 2 suggest the hypothesis that the reaction between RVV and beef serum is an enzymatic one, and may be interpreted as showing that RVV contains an enzyme which catalyzes the conversion of an inactive precursor(s) substance in beef serum to an active form. That the role of RVV is probably that of a catalyst is supported by observations (Fig. 1), that with other components at a constant concentration, the incubation time required to arrive at any arbitrary clotting time of the indicator plasma, say 15 seconds, is shorter the greater the concentration of RVV.

Over the range of RVV concentrations tested, there was also a slight difference in the shortest clotting times observed. Those incubation mixtures which required an increased incubation time to produce the most rapid clotting failed to develop as much activity as those mixtures with more RVV, where the production of activity was more rapid. Although this change in yield might be due to some component of the RVV acting stoichiometrically with the beef serum, the difference in yield is probably best ascribed to concomitant destruction of the formed active product. Such a phenomenon has been described in the conversion of prothrombin to thrombin.[13]

Much greater changes in the yield of active product were observed when the concentration of the beef serum was varied (Fig. 2). In these experiments, maximum activity, which varied sufficiently to give clotting times for the same plasma of from 8.5 to 17 seconds, was reached in each incubation mixture at approximately the same time. The effect of these changes in serum concentration is thus consistent with the hypothesis that beef serum contains an inactive clotting factor(s) that can be activated by an enzymatic reaction with RVV.

It is likely that the active component(s) of beef serum is a protein, although this conclusion rests, at present, only on inactivation of the beef serum component by heating to 56° C. and by retention of activity on dialysis. It has been shown that RVV can convert proaccelerin to accelerin,[14] and a component of beef serum involved in the reaction we have described could be proaccelerin (factor V). Present evidence is insufficient to establish if proaccelerin is involved here.

The appearance of trace amounts of thrombin in the incubation mixtures not containing added prothrombin (Tables I to III and Figs. 1 and 2) does not seem to be significant in our system, since the addition of even greater quantities of thrombin to either the substrate plasma or the complete incubation mixture fails to affect the observed end points.

The fact that full coagulant activity can develop in mixtures of RVV and beef serum in the absence of PE (Table II) indicates clearly that PE does not participate in the RVV-beef serum reaction. Nevertheless, there is very little conversion of prothrombin to thrombin by the RVV-beef serum product unless PE is present (Table IV). The PE would thus seem to be required just prior to or during the conversion of prothrombin to thrombin. Tissue thromboplastins do not require additional lipids for the rapid conversion of prothrombin[6]; however, perhaps they already contain the necessary phospholipids. The removal of lipids by alcohol-ether extraction from tissue thromboplastins destroys the activity of such preparations.[15]

It should be noted that when RVV alone, in high concentration, is added to recalcified phospholipid-enriched plasma, the clotting times observed are as short as those obtained here with the RVV-beef serum product. It is likely that the venom, when added to plasma, reacts with factors similar to those present in beef serum. We have shown (Fig. 1) that this reaction proceeds very rapidly with higher concentrations of the venom. Therefore, if a sufficient concentration of RVV is added to plasma, the reactions involving the RVV cease to be the rate-limiting reactions.

The identities of the factors in beef serum which are involved in the reaction with RVV were not ascertained in the experiments reported here. Further work is in progress directed toward identifying, separating, and concentrating these factors.

SUMMARY

1. The present work offers evidence that Russell's viper venom (RVV) reacts with some nondialyzable, heat-labile components of beef serum to form a product which, in the presence of phosphatidyl ethanolamine (PE), greatly accelerates the clotting of normal recalcified lipid-poor plasma and participates in the conversion of prothrombin to thrombin.

2. The phospholipid (phosphatidyl ethanolamine) greatly enhances the apparent activity of the Russell's viper venom-beef serum product. The presence of the phospholipid during the reaction between venom and beef serum, however, is not required.

3. The role of the Russell's viper venom may be that of an enzyme, acting on some protein component of the beef serum to produce a potent coagulant activity.

4. Further work is in progress to identify the clotting factors with which Russell's viper venom reacts.

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THE HEMOLYTIC EFFECT OF PRIMAQUINE
IX. Enzymatic Abnormalities in Primaquine-Sensitive Erythrocytes

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ERYTHROCYTES in approximately 10 per cent of the adult male Negro Americans are deficient in glucose-6-phosphate dehydrogenase,[1] and reduced glutathione,[2, 3] yet they have normal life spans unless challenged by a hemolytic drug.[4] Acute intravascular hemolysis results when these individuals are given an oral dose of 30 mg. of primaquine base daily.[4] Following an acute hemolysis, the glucose-6-phosphate dehydrogenase of the remaining erythrocytes does not rise to normal, nor does their reduced glutathione content[3]; nevertheless, these cells are resistant to further hemolysis by continuous daily administration of 30 mg. primaquine base[5] or an equivalent dose of a similar hemolytic drug. Many other drugs, and more are being discovered, can cause a "primaquine type" hemolysis when given to individuals with primaquine-sensitive erythrocytes.†[6-9]

The low reduced glutathione content of primaquine-sensitive erythrocytes falls precipitously when drug is given,[3] and this fall precedes the onset of the major hemolysis. In vitro studies have implicated reduced glutathione in the maintenance of erythrocyte integrity; e.g., the addition of organic mercurials or oxidizing agents to stored blood destroys most of the nonprotein sulfhydryl groups present and results in hemolysis.[10, 11]

The glutathione reductase in these erythrocytes has been studied,[12] under optimal concentrations of substrate and coenzyme, prior to hemolysis as

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†Sulfanilamide, phenacetin, acetanilid, sulfoxone, nitrofurantoin, naphthalene, and fava beans.

well as during hemolysis and recovery since this enzyme keeps most of the glutathione reduced according to the following reaction*:

$$(1)\quad GSSG + TPNH + H^+ \xrightarrow[\text{Reductase}]{GSSG} 2\ GSH + TPN^+.$$

METHODS

Since the reaction catalyzed by glutathione reductase is irreversible and stoichiometric, its activity can be assayed by determining the end product GSH.[13, 14]

Preparation of Dialyzed Hemolyzate.—1. Heparinized venous blood withdrawn at approximately 8 A.M. was centrifuged immediately at 1,800 g at 10° C. for 10 minutes in an International PR-2 refrigerated centrifuge. After removal of the plasma and buffy coat, the erythrocytes were resuspended in 3 volumes of cold 0.145 M NaCl, shaken well, and centrifuged for 15 minutes at 1,800 g at 10° C. The NaCl solution and remaining buffy coat were carefully taken off. The washed erythrocytes were transferred to a 125 ml. Pyrex glass-stoppered Erlenmeyer flask where they were hemolyzed by freezing and thawing 3 times, keeping the hemolyzate below 10° C. at all times. Five volumes of distilled water was then added. The mixture was shaken well and centrifuged for 90 minutes at 25,000 g (8° C.). The ghost-free supernatant solution was removed, placed in Visking cellulose bags (round diameter 28 mm., pore size 24 A.) containing a Kel-F marble, and dialyzed at 4° C. with constant motion for 16 to 18 hours against 0.067 M phosphate buffer in order to remove glutathione and hexose phosphates. The usual bag volume was approximately 35 ml., representing 6 ml. of washed erythrocytes. The buffer volume was 2,000 ml. Duplicate hemoglobin determinations were done on each hemolyzate before and after dialysis, and the enzyme activities were referred to the hemoglobin concentrations of dialyzate (GSH produced per gram hemoglobin dialyzate).

2. In studying the effect of daily administration of primaquine on glutathione reductase in hemolyzates, it was not feasible to perform dialyses each day. The hemolyzate for daily assay of glutathione reductase (Fig. 3) was prepared as follows.

Washed erythrocytes obtained as described above were frozen and thawed 3 times, and then 5 volumes of distilled water was added. At this point, 10 mg. of acid-washed Norit A was added per milliliter of diluted hemolyzate, and the mixture was shaken for 10 minutes at 10° C. The hemolyzate was centrifuged for 90 minutes at 25,000 g (8° C.) and the resulting supernatant was filtered through a medium sintered glass filter. This clear hemolyzate was then assayed for glutathione reductase. The treatment with Norit A served to remove all of the GSH and GSSG and had no deleterious effect on the glutathione reductase activity in the hemolyzate.

Standard Procedure for Assaying Glutathione Reductase.—Fourteen milliliter reaction mixtures in 0.2 M tris buffer, pH 7.4, contained 100 μM EDTA, 10 μM GSSG, and 12.6 μM of TPNH. Aliquots of dialyzed hemolyzate then were added to supply glutathione reductase, and the reaction mixtures were brought to final volume of 14 ml. with resin-treated distilled water. The reaction mixtures were incubated aerobically in a 37° C. water bath, and the reactions were stopped by the addition of 10 ml. 4.5 per cent metaphosphate solution. The protein-free filtrates were analyzed for GSH.[3]

Standard Procedure for Assaying Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconic Dehydrogenase.—Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were assayed in coupled systems according to the procedure described by Vennesland and Conn.[14] Both of these dehydrogenases specifically reduce TPN^+. Glutathione reductase present in the system utilizes the TPNH formed to reduce GSSG to GSH.

*TPN^+ = triphosphopyridine nucleotide; TPNH = reduced triphosphopyridine nucleotide; G-6-P = glucose-6-phosphate; 6-P-G = 6-phosphogluconate; GSH = reduced glutathione; and GSSG = oxidized glutathione.

Because the action of glutathione reductase is faster than either of the dehydrogenases, the formation of GSH provides a measurement of dehydrogenase activity in the coupled system when proper amounts of reactants are used.

Fourteen milliliter reaction mixtures in 0.07 M tris buffer, pH 7.4, contained 100 μM of EDTA, 10 μM GSSG, 7.5 μM of G-6-P (or 6-P-G), and 0.14 μM of TPN$^+$. Aliquots of dialyzed hemolyzate were added as a source of glucose-6-phosphate dehydrogenase (or 6-phosphogluconic dehydrogenase) and glutathione reductase. The reaction mixtures were brought to final volume of 14 ml. with resin-treated distilled water. As before, reactions were run aerobically in a 37° C. water bath, and the reactions were stopped by the addition of 4.5 per cent metaphosphate solution. Determinations for GSH then were performed on the protein-free filtrates.

Electrolytic reduction of appropriate blanks was done to determine the actual amount of GSSG available for enzymic reduction.

MATERIALS

Buffers.—1. Tris (hydroxymethyl) aminomethane was obtained as Sigma 121.* Stock 0.5 M solutions were prepared and brought to pH 7.4 with 1.0 N HCl.

2. Phosphate buffer (0.067 M, pH 7.4), used as dialyzing solution, was prepared by bringing 15.284 Gm. of Na_2HPO_4 and 3.495 Gm. KH_2PO_4 up to 2,000 ml. of solution with resin-treated distilled water.

Coenzymes.—TPN$^+$ and TPNH* were assayed both enzymatically and spectrophotometrically. Stock aqueous 14×10^{-5} M solutions of TPN$^+$ were prepared. The actual content of TPNH in the solid varied. Therefore, 7.5 mg. of TPNH was added per milliliter of 0.033 M phosphate buffer pH 8.4 to make approximately a 6.3×10^{-3} M solution. This solution was diluted 1:20 and assayed just prior to its use at 340 mμ using the extinction coefficient of 6.22×10^6 sq. cm./M.[15]

Substrates.—Oxidized glutathione was obtained from Schwarz Laboratories, Inc., and its purity was checked as previously described.[3] Aqueous 0.01 M solutions of GSSG were prepared.

Barium 6-phosphogluconate and sodium glucose-6-phosphate were obtained from the Sigma Chemical Company. Both were used as the sodium salt in aqueous 7.5×10^{-3} M solutions.

Chelating Agents.—Use of sodium ethylenediaminetetraacetic acid (EDTA, Versene) in the reactions was found necessary for optimum activity.[14] Stock 0.05 M solutions, pH 7.4, were prepared.

RESULTS

Glutathione Reductase, Glucose-6-Phosphate Dehydrogenase, and 6-Phosphogluconic Dehydrogenase in Hemolyzates of Primaquine-Sensitive and Normal Erythrocytes.—Glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic dehydrogenase were assayed using 1.0 ml. of dialyzed hemolyzate as the enzyme source with varying reaction times (Fig. 1). Hemolyzates prepared from primaquine-sensitive erythrocytes of 17 individuals had invariably increased glutathione reductase. In contrast, the glucose-6-phosphate dehydrogenase was decreased as shown previously,[1] and the 6-phosphogluconic dehydrogenase had the same activity as hemolyzates of normal erythrocytes under these optimal conditions.

This increase in glutathione reductase in hemolyzates of primaquine-sensitive erythrocytes was also found when increasing increments of dialyzed

*Obtained from the Sigma Chemical Company, St. Louis, Mo.

hemolyzates were used as the enzyme source with a fixed reaction time of 15 minutes (Fig. 2). From the initial straight-line portion of these representative curves, where enzyme activity is maximal, it can be seen that hemolyzates of primaquine-sensitive erythrocytes have 1.5 to 2 times as much glutathione reductase, but only $\frac{1}{8}$ to $\frac{1}{10}$ as much glucose-6-phosphate dehydrogenase, as do hemolyzates of normal erythrocytes.

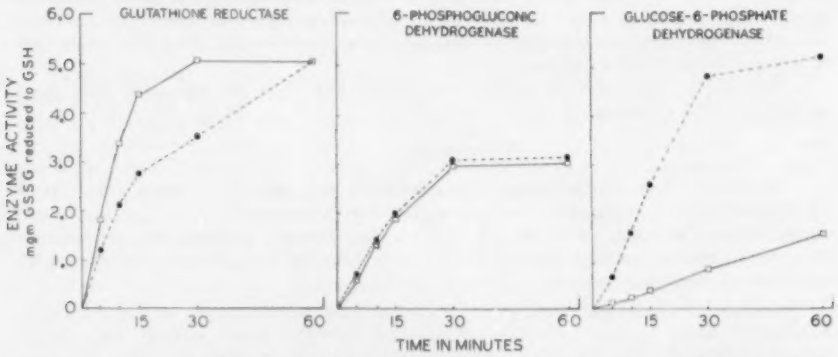

Fig. 1.—Time studies of glutathione reductase, 6-phosphogluconic dehydrogenase, and glucose-6-phosphate dehydrogenase in hemolyzates of primaquine-sensitive erythrocytes (□———□) and normal (●- - - -●) erythrocytes, using 1.0 ml. of dialyzed hemolyzate and varying the reaction times. In studies of glutathione reductase and glucose-6-phosphate dehydrogenase, GSSG was the limiting reactant, while 6-PG was the limiting factor in the 6-phosphogluconic dehydrogenase reactions.

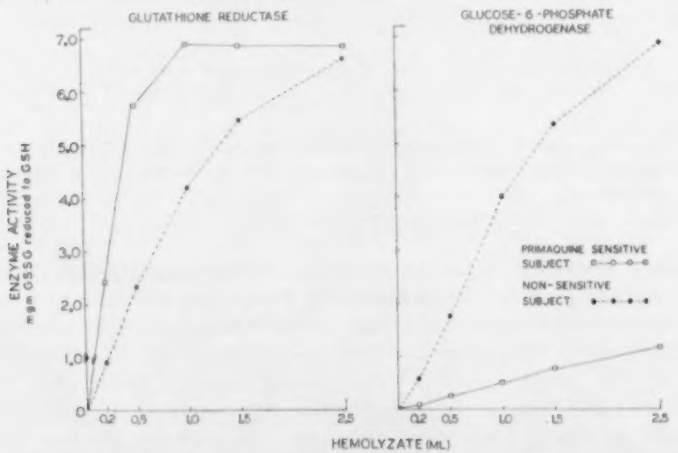

Fig. 2.—Comparison of glutathione reductase and glucose-6-phosphate dehydrogenase using increasing amounts of hemolyzates of primaquine-sensitive erythrocytes (□———□) and normal erythrocytes (●- - - -●). In these experiments, the 14 ml. reaction mixtures contained 13 μM GSSG and were incubated for 15 minutes.

The Effect of Primaquine on Glutathione Reductase of Hemolyzates.—The glutathione reductase in hemolyzates (prepared by treatment with acid-washed Norit A) from primaquine-sensitive and normal individuals was determined

each morning during a period of daily oral administration of 30 mg. primaquine base (Fig. 3). There was no change in the glutathione reductase in hemolyzates of either normal or primaquine-sensitive erythrocytes during this period.

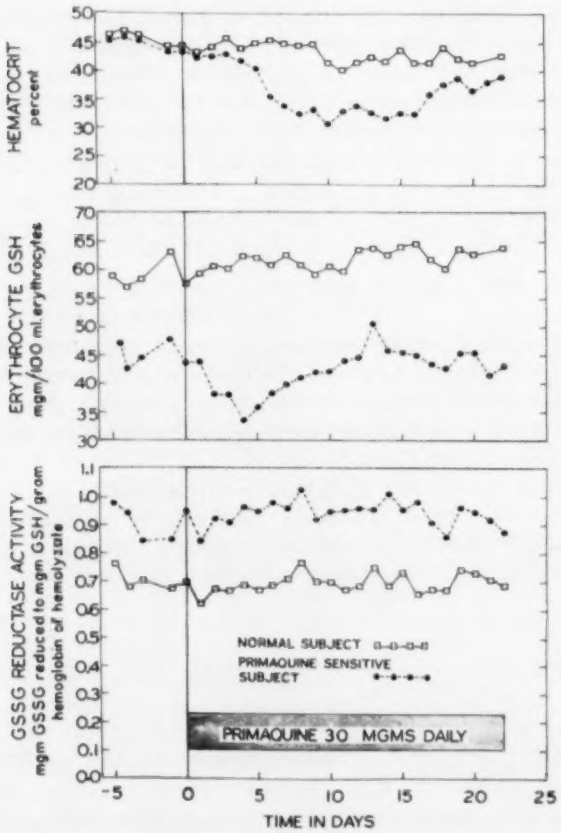

Fig. 3.—Effect of primaquine administration on glutathione reductase in hemolyzates, GSH content of erythrocytes, and hematocrit in a primaquine-sensitive and a normal individual. To assay for glutathione reductase in these experiments, 14 ml. reaction mixtures containing 1.0 ml. of Norit-treated hemolyzate and 13 μM of GSSG were incubated for 15 minutes at 37° C.

DISCUSSION

A. In a previous communication from this laboratory it was reported that the glucose-6-phosphate dehydrogenase in hemolyzates of primaquine-sensitive erythrocytes was markedly decreased, whereas the 6-phosphogluconic dehydrogenase and the glutathione reductase were normal.[1] Subsequent investigation of these reactions at 37° C. has shown that maximal enzyme activity occurs with a sixfold increase in substrate, GSSG, and a corresponding increase in the

coenzyme, TPNH. These studies revealed that hemolyzates of primaquine-sensitive erythrocytes consistently had substantial elevations of glutathione reductase in the order of 1.5 to 2 times normal. Our previous finding that glucose-6-phosphate dehydrogenase in primaquine-sensitive erythrocytes is markedly deficient, in the order of $\frac{1}{8}$ to $\frac{1}{10}$ of normal, has been confirmed.* Because the deficiency of glucose-6-phosphate dehydrogenase is several fold greater (Fig. 2) than the increase in glutathione reductase in primaquine-sensitive erythrocytes, we have postulated that the deficiency of glucose-6-phosphate dehydrogenase is the primary enzymic abnormality.

This increase in glutathione reductase may be a factor enabling the primaquine-sensitive erythrocyte to complete a normal life span under usual conditions; however, apparently it is not sufficient to protect the cell from hemolysis when it is exposed to the in vivo action of the hemolytic drug.

B. There are now 3 known biochemical abnormalities in primaquine-sensitive erythrocytes: (1) low GSH but normal GSSG content[3]; (2) deficient glucose-6-phosphate dehydrogenase; and (3) increased glutathione reductase. On the basis of the two known functions of glutathione reductase we have proposed two means by which increased glutathione reductase may partially compensate for the decrease in both glucose-6-phosphate dehydrogenase and reduced glutathione.

In our studies of hemolyzates, glutathione reductase rapidly oxidizes TPNH to TPN^+ (Reaction 1). TPN^+ is reduced to TPNH by glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase (Reactions 2 and 3) which successively catalyze the oxidative reactions in the hexosemonophosphate shunt.

$$(2)\quad \text{G-6-P} + TPN^+ \xrightarrow[\text{Dehydrogenase}]{\text{G-6-P}} \text{6-P-G} + TPNH + H^+$$

$$(3)\quad \text{6-P-G} + TPN^+ \xrightarrow[\text{Dehydrogenase}]{\text{6-P-G}} \text{Pentose phosphate} + CO_2 + TPNH + H^+$$

The hexosemonophosphate shunt is a TPN^+ dependent pathway of direct glucose oxidation which is probably active in the mature human erythrocyte.[17]

It is postulated that increased glutathione reductase may partially compensate for deficient glucose-6-phosphate dehydrogenase by regenerating the available coenzyme, TPN^+, at a rate that may allow the deficient enzyme to work more effectively† by mass action law (Reaction 2). A similar explanation has been proposed for the pyruvate stimulation of the hexosemonophosphate shunt.[18]

*The deficiency of glucose-6-phosphate dehydrogenase was also confirmed by Dr. E. S. G. Barron using hemolyzates and whole red blood cells as the source of this enzyme in experiments with the Warburg apparatus.[16]

†Theoretically, also, excess glutathione reductase could deplete erythrocytes of TPNH, a coenzyme[19] essential for reductive bio-synthetic processes, and, thereby, exert a detrimental effect.

Reduced glutathione has been shown to protect or activate certain sulfhydryl enzymes and proteins,[20, 21] and this may account for the importance of GSH in maintaining the integrity of the erythrocyte.[10, 11] During cellular activity, GSH may be oxidized either enzymatically[14, 21, 22] or nonenzymatically[23] to GSSG. Glutathione reductase then reduces the GSSG formed. Increased glutathione reductase may thereby make more GSH available to essential sulfhydryl enzymes and proteins in the GSH deficient erythrocyte.

C. In mammalian tissues the distribution of glutathione reductase seems to parallel that of enzymes of the hexosemonophosphate shunt.[24, 25] Increased glutathione reductase in cells deficient in the initial enzyme of the hexosemonophosphate shunt is additional suggestive evidence of the dependent relationship between the two systems. It seems likely that glutathione reductase acts as the electron transporting enzyme for the hexosemonophosphate shunt in the human erythrocyte.

D. Administration of primaquine does not appear to affect glutathione reductase in hemolyzates of either normal or primaquine-sensitive erythrocytes since the enzyme was unchanged during primaquine-induced hemolysis and recovery (Fig. 3). This absence of change in glutathione reductase indicates that it is not the site of hemolytic drug action, nor is it primarily concerned in the mechanism by which primaquine-sensitive erythrocytes become resistant to continued administration of the same dose of hemolytic drug.

SUMMARY

1. Hemolyzates of primaquine-sensitive erythrocytes have 1.5 to 2 times as much glutathione reductase as do hemolyzates of normal erythrocytes. In contrast, primaquine-sensitive erythrocytes have normal amounts of 6-phosphogluconic dehydrogenase, but only ⅛ to 1/10 normal amounts of glucose-6-phosphate dehydrogenase.

2. Because the deficiency in glucose-6-phosphate dehydrogenase is several fold greater than the increase in glutathione reductase, it is postulated that the deficiency of glucose-6-phosphate dehydrogenase is the primary enzymic abnormality in primaquine-sensitive erythrocytes, and that increased glutathione reductase represents an attempt by these erythrocytes to compensate for deficiencies in glucose-6-phosphate dehydrogenase and GSH by: (1) providing greater availability of the obligatory coenzyme, TPN^+, and allowing the deficient glucose-6-phosphate dehydrogenase to work at its maximum rate, and (2) regenerating GSH more efficiently after its oxidation is brought about in the erythrocyte, thereby partly overcoming the deficiency in GSH.

As yet, we have been unable to determine which, if either, of these proposed compensatory actions is actually operative.

3. Oral primaquine administration does not affect glutathione reductase in hemolyzates of either normal or primaquine-sensitive erythrocytes. Therefore, the primary site of action of the hemolytic agent must lie elsewhere.

4. Primaquine-sensitive erythrocytes, deficient in GSH and glucose-6-phosphate dehydrogenase, have normal life spans unless they are challenged by

hemolytic drug. Increased glutathione reductase and other undiscovered abnormalities may help maintain the integrity of the erythrocytes in the face of continuously existing deficiencies.

We believe that further abnormalities of enzymes, coenzymes, and substrates will be shown to exist in this condition as manifestations of deficiencies and compensatory responses. Our present studies concern the search for these abnormalities.

The advice and interest of Dr. Birgit Vennesland is gratefully acknowledged.

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A METHOD FOR MEASURING THE TURNOVER OF FLUID IN THE PLEURAL AND OTHER SEROUS CAVITIES

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THERE is very little quantitative data on rates of turnover of fluid in the serous cavities. Starling and Tubby[1] measured the net changes of volume in pleural cavity after introduction of a known quantity of fluid at intervals of up to 6 hours. Courtice and Simmonds[2] were able to obtain additional information by labeling the albumin with T1824 of the plasma injected into the pleural cavity of cats and rabbits and measuring the concentrations of this labeled protein in the circulation and lymphatic channels.

This paper describes an adaptation of the method of Courtice and Simmonds which yields quantitative information on the movement of fluid in the pleural cavity of unanesthetized animals. An illustration of the use of the method in the dog's pleural cavity will be given. The method appears to be directly applicable to studies on other serous cavities.

THEORY

The methods used in the present experiments to follow the movement of pleural fluid stems from the observation of Courtice and Simmonds[2] that labeled protein introduced into the pleural cavity is removed exclusively by the lymphatics. This conclusion was based on: (1) the failure of plasma labeled with T1824 to enter the circulating blood from the pleural cavity after the thoracic and right lymphatic duct had been ligated and (2) the high concentrations of dye found in the lymphatics when they were cannulated. Assuming that this holds true in dogs, the removal of the labeled protein from the pleural cavity would be a measure of the volume of flow in the lymphatic vessels, hereafter called the lymphatic flow. If the concentration of labeled protein in the fluid entering the lymphatics were known, the lymphatic flow could readily be calculated. The fluid entering the lymphatics is derived from the pleural fluid, and the question at issue is whether the pleural fluid is subjected to any ultrafiltration while entering the lymphatic capillaries. This would cause the concentration of the protein that entered the lymphatics to be different from that in the pleural fluid. It must be emphasized that subsequent concentration changes in the lymph channels do not introduce any error in the method adopted here, provided that all the labeled protein absorbed from the pleural cavity reaches the circulating blood plasma. The evidence against ultrafiltration by the pleural lymphatic capillaries is, first, that large particles such as red and white blood cells readily leave the pleural cavity in the lymphatics. Indeed, it will be shown that the rate of absorption of reticulocytes is not appreciably different from that of labeled protein.[3] It is hardly conceivable that openings permitting the free entry of cells of 5 to 10 μ could have a sieving

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effect on proteins with a maximum axial diameter of little more than a thousandth of this size. Second, if the lymphatic capillaries were the site of ultrafiltration, it is difficult to see how the force could be provided to draw fluid into the lymphatics from the pleural cavity against the partial osmotic pressure created by the holdback of proteins. This force would necessitate a pressure considerably lower in the lymphatics than in the pleural cavity. No evidence exists that this occurs, and, in any case, since the lymphatics are not rigid such a pressure difference could not be established. Third, in the methods to be described, the lymphatic flow from the pleural cavity and the fluid entry to the pleural cavity directly from the blood are assessed independently. From these measurements the total changes in the volume of pleural fluid over a period of 6 to 10 hours can be calculated. The calculated volume of pleural fluid present at the end of an experiment and that obtained by direct measurement during life, or after killing the dog, was found to be in close agreement by the method of dilution. Fluid was always found in both pleural cavities in approximately equal amounts at the end of an experiment, though it had been put initially into one or other cavity. The concentration of label was the same on both sides showing that functionally the pleural cavities behave as a single unit. The lymphatic flow which is measured by our method is thus from both pleural cavities.

If sieving of protein was a feature of lymphatic drainage, the calculated volume would be consistently in error by an amount dictated by the "reflectivity coefficient" of the membrane[4] as compared with the volume measured directly.

Finally, there is much evidence[5] that the lymphatic system is adapted for the very purpose of removing large molecular weight substances, particulate matter, and cells.

We can assume, therefore, that the concentration of labeled protein entering the pleural lymphatics is the same as that in the pleural fluid.

As there is no appreciable loss of the label (T1824 or I^{131}) from the body over the period of the experiments, the amount of label originally introduced into the pleural cavity is divided between that remaining in the pleural cavity and the amount that has become extrapleural. When the label is discharged from the lymphatics into the blood, it circulates in the plasma and slowly leaks out into the extravascular space. The plasma volume and rate of loss of the protein label into the extravascular space can be measured after intravenous injection of the label. For short periods this rate of loss of label into the extravascular space is linear with time. We have confirmed that T1824 and I^{131} albumin give identical values for plasma volume and also, over the period of time relevant to our experiments, the same rate of loss into extravascular space. We may, therefore, determine all our parameters simultaneously, using one label (I^{131} albumin) in the pleural cavity and a second (T1824) for determination of the plasma volume and the rate of extravascular loss of pleural label.

To measure the lymphatic flow from the pleural cavity, we need to know the concentration of the label in the pleural fluid and the amount of label which is extrapleural.

The amount of extrapleural label (Qep) is given by

$$Qep = PV\ (Cp + a \int_{o}^{x} Cp \cdot dt) \qquad (1)$$

where PV = plasma volume; C_p = plasma concentration of pleural label; a = the fraction of intravenous label leaving the plasma and entering the extravascular space per unit time.

The validity of this equation has been tested by direct measurement of the amount of label remaining in the pleural cavity at the end of an experiment. This amount when subtracted from the amount originally injected gives the actual amount of label which has become extrapleural and has agreed closely with the calculated figure.

The lymphatic flow $\frac{dV_L}{dt}$ is, therefore:

$$\frac{dV_L}{dt} = \frac{1}{Cpl} \cdot \frac{dQep}{dt} \qquad (2)$$

where Cpl = pleural label concentration in pleural fluid.

The volume of pleural fluid (Vpl) at any time is given by

$$Vpl = \frac{Qpl}{Cpl} \tag{3}$$

where Qpl = quantity of pleural label in the pleural fluid.

Differentiating equation 3,

$$\frac{dVpl}{dt} = d\,\frac{Qpl/Cpl}{dt} = \frac{1}{Cpl} \cdot \frac{dQpl}{dt} - \frac{Qpl}{Cpl^2} \cdot \frac{dCpl}{dt}. \tag{4}$$

The rate of change of pleural volume with time $\frac{dVpl}{dt}$ is composed of the algebraic sum of two components: (1) the rate of unidirectional loss of fluid through the valved lymphatics $\frac{dV_L}{dt}$ (i.e., the lymphatic flow) and (2) the rate of direct movement of fluid between blood and pleural cavity. This may be from pleural cavity into the blood or in the opposite direction and may be designated $\frac{dV_{NL}}{dt}$ (nonlymphatic flow).

Thus,

$$\frac{dV_L}{dt} = \frac{dV_{NL}}{dt} - \frac{dV_L}{dt}. \tag{5}$$

The convention is thus established of giving $\frac{dV_{NL}}{dt}$ a positive sign if it represents a movement of fluid into the pleural cavity from the blood and a negative sign when it represents movement of fluid out of the pleural cavity into the blood.

Rearranging equation 5,

$$\frac{dV_{NL}}{dt} = \frac{dVpl}{dt} + \frac{dV_L}{dt} \tag{6}$$

and substituting equation 4 in equation 6,

$$\frac{dV_{NL}}{dt} = \left[\frac{1}{Cpl} \cdot \frac{dQpl}{dt} + \frac{dV_L}{dt}\right] - \frac{Qpl}{Cpl^2} \cdot \frac{dCpl}{dt}. \tag{7}$$

Since the loss of label from the pleural cavity is accomplished by a simultaneous and equal increase in extrapleural label (Qep), then

$$\frac{dQpl}{dt} = -\frac{dQep}{dt}. \tag{8}$$

Multiplying both sides of equation 8 by $\frac{1}{Cpl}$ we obtain:

$$\frac{1}{Cpl} \cdot \frac{dQpl}{dt} = -\frac{1}{Cpl} \cdot \frac{dQep}{dt}. \tag{9}$$

Since in equation (2) $\frac{dV_L}{dt} = \frac{1}{Cpl} \cdot \frac{dQep}{dt}$, then, substituting this in equation 9,

$\frac{1}{Cpl} \cdot \frac{dQpl}{dt} = -\frac{dV_L}{dt}$.

The left hand side of this is one of the terms inside the bracket in equation 7 which, therefore, becomes $\left[-\frac{dV_L}{dt} + \frac{dV_L}{dt}\right]$ i.e., $= 0$.

Therefore, the nonlymphatic flow is given by

$$\frac{dV_{NL}}{dt} = -\frac{Qpl}{Cpl^2} \cdot \frac{dCpl}{dt}. \tag{10}$$

The magnitude of $\frac{dV_{NL}}{dt}$ depends mainly on the rate of change of the pleural label concentration. If this concentration is falling then $\frac{dV_{NL}}{dt}$ is positive showing a net movement of fluid from the blood into the pleural cavity. If the concentration of pleural label does not change, then $\frac{dV_{NL}}{dt} = 0$ and there is no net movement between blood and pleural cavity. If the concentration of pleural label is rising then $\frac{dV_{NL}}{dt}$ is negative and the net movement of fluid is from the pleural cavity to the blood.

The rate of lymphatic flow from the pleural cavity, the rate of nonlymphatic fluid movement, and the volume of pleural fluid may be measured at any time during the course of an experiment by means of these equations.

There are three potential sources of error in the above equations. First, we do not usually make allowance for the time taken for the labeled protein to traverse the dead space of the pleural lymphatics. In the bulk of the experiments the concentration of the labeled protein in the pleural fluid is changing slowly and neglecting dead space makes the results of the first 1 to 2 hours invalid but introduces only a small error (1 to 4 per cent) in the later calculations. When rapid changes occur in the concentration of the pleural label the error is more serious and is allowed for. Second, an error could occur from labeled protein being absorbed through the lymphatics into the blood stream and then re-entering the pleural cavity through the pleural capillaries. Except in prolonged experiments this is a negligible source of error because the usual concentration of the pleural label in the pleural fluid is about 25 times higher than its concentration in the plasma. In the more prolonged experiments, the entry of the intravenous label used for the plasma volume determination into the pleural cavity, may be used to correct for the re-entry of the pleural label. Finally, in experiments where rapid fluid transfer occurs, correction must be made for the resulting change in plasma volume.

METHODS

The experiments were carried out on trained mongrel dogs weighing 12 to 20 kilograms. The sixth or seventh intercostal space in the posterior axillary line was infiltrated with local anesthetic. A 14 S.W.G. needle connected to a water manometer was inserted until a fluctuating subatmospheric pressure indicated that the pleural cavity had been entered. A catheter was introduced through the needle, which was then withdrawn. The catheter was of fine polyethylene tubing (Clay Adams P.E. 90) with several side openings made in its terminal 5 cm. and was coated with silicone (Beckman Desicote). The outer end of the catheter was occluded with a spigot to prevent air from entering the pleural cavity. A similar catheter (Clay Adams P.E. 50) introduced through a 17 S.W.G. needle was placed in a foreleg vein. Through the chest catheter 10 to 15 ml. per kilogram of plasma containing a known amount of T1824 or iodinated^{131} bovine albumin was placed in the pleural space.

Collection of Fresh Plasma.—Four-tenths milliliter of a 1 per cent heparin solution was put into siliconed cellulose nitrate centrifuge tubes of 50 ml. volume and allowed to dry. Blood was collected from the carotid arteries of anesthetized dogs into these tubes, through a siliconed polyethylene cannula, and immediately centrifuged at 8,000 r.p.m. for 5 to 10 minutes in a refrigerated centrifuge. The plasma was separated and, after adding either T1824 or iodinated^{131} bovine albumin, was injected into the pleural cavity within 30 minutes of withdrawal from the donor.

Samples of pleural fluid and blood were obtained at regular intervals and the concentration of the protein label measured. All apparatus, except the catheters, was sterilized by dry heat. The catheters were sterilized by immersion in benzalkonium for 24 hours before use.

T1824 Estimations.—The T1824 was obtained from Warner-Chilcott Laboratories. It was estimated in plasma and pleural fluid by the acetone extraction method of Chinard.[6] If necessary the extract was diluted with a 3:1 mixture of acetone and 0.9 per cent saline. The absorption was read at 620 mμ in a Beckman DU spectrophotometer.

Estimation of Iodinated Albumin.—I^{131}-labeled bovine albumin was prepared for us by Charles E. Frosst & Company, Montreal. The average iodination was 1 to 2 atoms per molecule and the material was received with a specific activity of 10 μc per milligram of protein. It was exhaustively dialyzed against 0.9 per cent saline until completely free of inorganic iodide. Radioactivity was measured with a mica end-window G.M. tube (Tracerlab T.G.C. 2) on 0.2 ml. samples pipetted into aluminum dishes. One drop of 10 per cent Carbowax 5000 was added to prevent cracking, and the dishes were then dried under an infrared lamp. Appropriate corrections were made for self-absorption and radioactive decay.

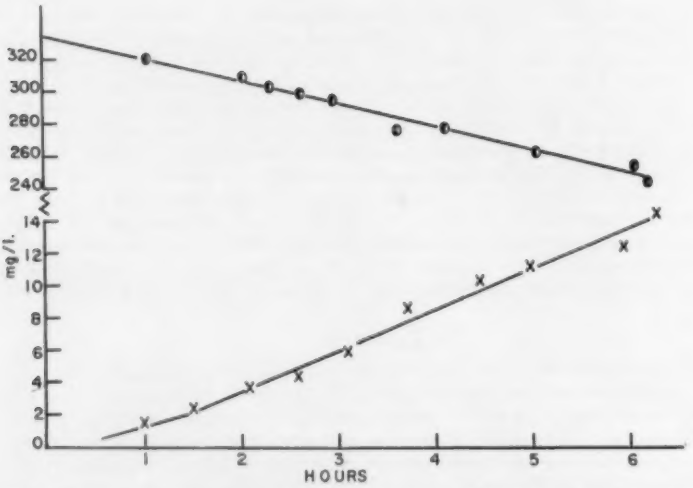

Fig. 1.—Resting unanesthetized dog, 19.1 kilograms. Two hundred and ten milliliters of fresh heparinized plasma containing 69.93 mg. of T1824 was injected into the left pleural cavity at time zero.

Key: ◐———◐ = concentration of T1824 in pleural fluid. ×———× concentration of T1824 in circulating plasma. Ordinates = milligrams of dye per liter. Abscissas = time in hours.

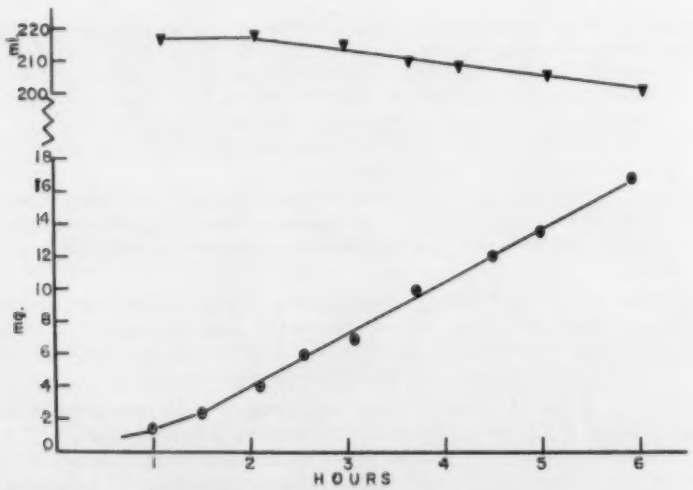

Fig. 2.—The same experiment as Fig. 1.

Key: ▼———▼ = calculated volume of fluid in the pleural cavity. ⊕———⊕ = extrapleural label in milligrams calculated from equation 1. Ordinates = (a) milligrams of dye; (b) milliliters of fluid. Abscissas = time in hours. (Note: the smoothed data in this and the previous figure are not used in calculations.)

RESULTS

Fluid Movement After Intrapleural Injection of Fresh Heparinized Plasma in Dogs at Rest.—The results of a typical experiment are shown in Figs. 1, 2, and 3. In this case 210 ml. of fresh heparinized plasma containing 69.93 mg. of T1824 was injected into the pleural cavity of an unanesthetized dog weighing 19.1 kilograms. It can be seen in Fig. 1 that the concentration of dye in the pleural fluid gradually fell from an initial value of 334 mg./L. to 246 mg./L. after 6 hours. At the same time the plasma concentration rose to 14.1 mg./L. which was only 5.72 per cent of the pleural concentration at this time. The lag in the rise in the plasma concentration in the first 1½ hours discussed earlier can be seen. Fig. 2 shows the amount of extrapleural T1824 (Qep) calculated by equation 1, and the volume of pleural fluid calculated by equation 3. The volume of fluid declined slowly during the course of the experiment.

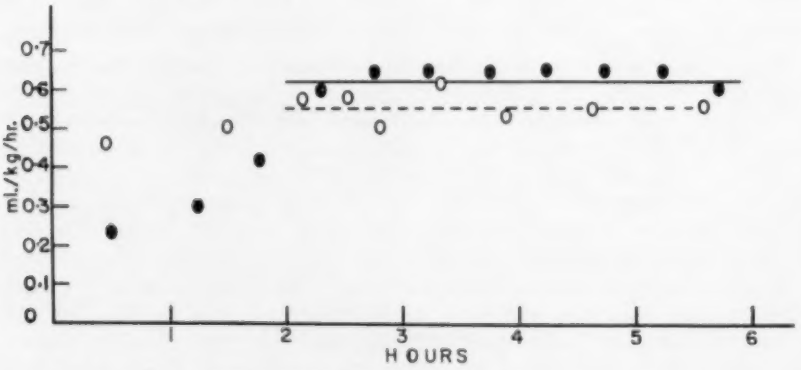

Fig. 3.—The same experiment as Figs. 1 and 2.
Key: ●——● = lymphatic flow $\left(\frac{dV_L}{dt}\right)$ calculated from equation 2 smoothed data; ○- - - - -○ = nonlymphatic flow $\left(\frac{dV_{NL}}{dt}\right)$. Calculated from equation 10. Ordinates = milliliters per kilogram hour. Abscissas = time in hours.

The lymphatic and nonlymphatic flows calculated from equation 2 and equation 10 are shown in Fig. 3. In this figure the rates of flow have been smoothed. There was a steady lymphatic drainage of 0.63 ml./Kg. hr. which was almost compensated for by an entry into the pleural cavity of 0.54 ml./Kg. hr. from the blood. Thus, the volume of pleural fluid was decreasing in this case by only 0.09 ml./Kg. hr.

DISCUSSION

This method has been extensively used in the study of pleural fluid movements. Final volumes of pleural fluid calculated from the equations given rarely differed from the volumes actually found by the method of dilution or at autopsy by more than 5 per cent. The application of this method to the study of effects of various procedures on pleural fluid movements will be described in a subsequent paper.[3]

SUMMARY

A method is described for measuring quantitatively the movement of fluid into and out of the pleural cavity by lymphatic and nonlymphatic channels in the unanesthetized dog. The method is applicable to other serous cavities and involves measurement of the changes in concentration of a labeled protein both in the fluid of the cavity and in the circulating blood plasma, after its injection into the cavity.

We would like to express our thanks to Dr. Hugh E. Burke for many helpful discussions.

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THE INFLUENCE OF SEDATIVE AGENTS UPON RENAL HEMODYNAMICS

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IT IS often desirable to use sedative drugs in the preparation of children for renal hemodynamic studies. However, no data are available to indicate the extent to which the commonly employed soporific agents influence renal function in man.

In 1939, Smith[1] stated that studies which involved anesthetic agents and surgical procedures were not likely to yield useful information concerning the normal physiology of the kidney. Corcoran and Page,[2] producing anesthesia by the intraperitoneal administration of pentobarbital sodium, found no change in the Diodrast clearance, tubular secretory capacity, or inulin clearance in the great majority of their dogs. They did find an increase in arterial blood pressures, however. An occasional dog demonstrated acute failure of renal function, suggesting that they were on the threshold of toxicity. Glauser and Selkurt,[3] studying the influence of barbiturates on renal function in dogs, noted that while experiments of short duration (1 to 2 hours) did not significantly affect filtration rate or plasma flow, extended periods of study (over 3 hours) under barbiturate sedation were associated with reduction in renal plasma flow and tubular excretory capacity for para-aminohippurate. Recent studies by Handley and Moyer[4] of renal function in dogs following sedation with morphine show a pronounced fall in mean blood pressure as well as an abrupt and sustained depression of glomerular filtration rate, renal plasma flow, and maximal rate of reabsorption of dextrose by the renal tubules. They felt that the proportional decrease in glomerular filtration rate, tubular maximum reabsorption of glucose, and renal plasma flow indicated that a variable number of nephrons became inactive after morphine due to renal vasoconstriction.

Papper and associates,[5] in studying the effect of morphine on the excretion of water and solute in normal adult men, demonstrated a decrease in urine flow unaccompanied by a rise in urine osmolarity, but it was associated with a decrease in endogenous creatinine clearance and the rate of total solute excretion. This would indicate that the decrease in the rate of diuresis occurred without evident release of antidiuretic hormone.

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TABLE I. THE INFLUENCE OF SEDATIVE DRUGS

SUBJECT INITIAL	S.A.	DRUG DOSE MG./M.²	DEGREE OF SEDATION*		MEAN BLOOD PRESSURE			GLOMERULAR FILTRATION RATE† (C.C./MIN.)		
			D_1	D_2	C	D_1	D_2	C	D_1	D_2
Meperidine Studies										
1. C. S.	0.91	70	1	1	98	98	105	151	143	119
2. C. B.	1.46	70	2	1	77	70	78	146	113	139
3. C. G.	1.15	70	2	2	91	92	91	133	82	118
4. D. P.	0.97	50	2	1	84	83		178	167	
Mean					87	85	91	152	126	125
Per cent C						98	105		83	82
Phenobarbital Studies										
5. A. M.	0.88	100	3	2	82	73	74	142	126	136
6. E. F.	1.17	100	1	1	100	92	97	113	139	164
7. A. F.	1.31	70	3	3	71	70	70	81	82	86
8. L. E.	1.13	106	2	1	83	83	83	129	135	95
9. B. U.	0.85	150	3	2	96	94	84	120	96	96
10. B. J.	1.30	120	2	2	79	91	89	107	109	95
11. J. Mc.	1.15	150	2	2	93	91	90	156	147	182
Mean					86	85	84	121	119	122
Per cent C						99	98		98	101
Chloral Hydrate Studies										
12. R. G.	1.15	1,500	2		79	77		101	101	
13. R. A.	0.82	1,500	2		70	66		103	92	
14. A. B.	0.69	1,900	2		92	101		125	120	
15. H. F.	1.06	2,000	2	1	87	94	96	108	98	99
16. F. T.	0.64	2,000	3	4	73	70	66	130	132	121
17. Mc. D.	0.95	1,700	3	2	96	85	82	189	195	169
18. C. H.	0.86	1,750	3	3	82	78	82	178	122	112
Mean					83	82	82	133	123	125
Per cent C						99	99		92	94

*Key: 1 = drowsy, some dozing; 2 = light sleep most of period; 3 = slept soundly; 4 =
†Corrected to 1.73 M.² surface area.

The present studies were undertaken to determine the influence of 3 sedative agents (meperidine, chloral hydrate, and phenobarbital) on glomerular filtration rate, renal blood flow, water excretion, and the clearance of sodium and potassium in children. In addition, the influence of chloral hydrate and phenobarbital on tubular excretory capacity for para-aminohippurate was measured.

MATERIAL AND METHODS

The subjects selected for study were either convalescent or awaiting elective surgical procedures. Their ages ranged from 4 to 13 years. All were in a state of normal hydration and nutrition. Each patient served as his own control (Period C) following which the drug to be studied was given. As soon as the subject showed evidence of sedation, the observations were repeated (Period D_1). A third set of observations (Period D_2) was obtained 30 minutes to one hour after the second study period. The quantity of sedative agent administered and the degree of sedation at the time of study are indicated in Table I. The degree of sedation was graded from 1 to 4 as indicated in the key to Table I.

The details of clearance technique used in this laboratory have been described previously.[6] Glomerular filtration rate was measured by the clearance of inulin and renal plasma flow by the low-level clearance of sodium para-aminohippurate. Tubular excretory capacity was measured by the high-level excretion of para-aminohippurate. Sodium and potassium were determined using a Beckman DU flame photometer. Mean blood pressure was calculated as the diastolic plus one-third of the pulse pressure.

ON RENAL HEMODYNAMICS IN CHILDREN

EFFECTIVE RENAL BLOOD† FLOW (C.C./MIN.)			WATER EXCRETION† (C.C./MIN.)			CLEARANCE POTASSIUM† (C.C./MIN.)			CLEARANCE SODIUM† (C.C./MIN.)			Tm PAH†		
C	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2	C	D_1	D_2
1,062	989	984	20	21	19	17	10	10	1.8	1.6	1.4			
1,126	816	991	16	1	14	8	4	6	1.6	0.5	0.9			
1,681	788	1,105	17	1	2	10	4	2	3.5	1.5	1.3			
1,876	1,457		9	2		19	8		1.2	1.3				
1,436	1,012	1,026	15	6	12	13	6	6	2.0	1.2	1.2			
	70	71		40	80		46	46		60	60			
1,884	1,764	1,695	10	9	15	17	17	13	1.3	1.4	1.3			
1,141	1,102	942	15	9	5	24	10	13	2.0	2.2	1.8			
645	605	570	10	7	5	28	15	12	1.5	1.4	1.4			
1,182	1,158	671	7	7	7	21	18	19	1.0	2.0	1.8			
			3	5	3							75	54	60
			3	11	7							35	37	31
			3	7	11							82	81	71
1,213	1,157	970	7	8	8	22	15	14	1.4	1.7	1.6	64	57	54
	95	80		114	114		68	64		121	114		89	84
1,341	1,130		16	12		22	24		1.2	1.1				
1,226	1,020		13	2		18	21		1.5	0.7				
855	652		1	10		26	8		0.7	0.5				
1,403	1,485	1,165	7	19	15	10	12	15	2.1	3.8	4.1			
			4	25	26							95	40	19
			6	13	3							101	89	61
			4	12	18							95	78	78
1,206	1,072		7	13	16	19	16		1.4	1.5		97	69	52
	89			186	229		84			107			71	54

responded minimally to painful stimuli.

RESULTS

Reference to Table I reveals that none of the sedative drugs studied had any significant or consistent influence upon the mean blood pressure. There were also no significant changes in pulse rate. The effective renal blood flow was moderately decreased by all 3 soporific agents, but not as much by phenobarbital and chloral hydrate as by meperidine. While no significant changes in glomerular filtration rate were observed, there was a modest and consistent reduction following meperidine sedation. The rate of water excretion was extremely variable. Following meperidine sedation, 3 of the 4 patients showed a marked reduction in the rate of water excretion while 1 demonstrated no change. Phenobarbital did not appear to influence the rate of water excretion to any significant degree while there was an apparent increase in the rate of water excretion following chloral hydrate. The clearance of potassium was significantly reduced (Period D_1) by both meperidine and phenobarbital and showed little tendency to recover (D_2) during the period of observation. The clearance of potassium was not influenced by chloral hydrate. Although the clearance of sodium was not significantly influenced by any of the sedative agents studied, the changes noted were less with chloral hydrate and phenobarbital and greater following meperidine.

The maximal tubular excretory capacity for para-aminohippurate was reduced in patients sedated with both chloral hydrate and phenobarbital, but to a lesser extent in the patients sedated with phenobarbital.

DISCUSSION

On the basis of the data obtained, it would appear that in the usual soporific doses chloral hydrate, phenobarbital, and meperidine are all satisfactory agents for the preparation of children for studies of gross renal function. However, individual variations in responsiveness are marked and may influence the interpretation of the patient's renal status when definitive and precise information is desired. The soporific effect of all 3 drugs is approximately similar; however, the tendency for meperidine to reduce water and electrolyte excretion and renal blood flow makes it the least desirable of the 3 drugs studied. The depression of tubular excretory capacity appeared to be more marked following chloral hydrate sedation than after phenobarbital sedation. Although the changes in renal function following the administration of soporific agents as noted in these studies are generally not marked, they demonstrate a factor which must be considered in the evaluation of renal function studies.

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A NEW TECHNIQUE FOR SIMULTANEOUS ESTIMATION OF TOTAL BODY WATER AND TOTAL EXCHANGEABLE BODY SODIUM USING RADIOACTIVE TRACERS

The Results in 13 Normal Men

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IN STUDIES on fluid and electrolyte metabolism it is frequently desirable, particularly in disease states, to measure total body water (TBW) and total exchangeable body sodium (TEBS) simultaneously. Tritium water (HTO) has many advantages for estimation of TBW[1] and the availability of reliable liquid scintillation counters makes the assay of this radioisotope of hydrogen feasible. Previously published methods using liquid scintillation techniques have certain disadvantages[2, 3] and do not permit direct measurement of HTO in the presence of radiosodium.[22]

In the present report an improved method is described for simultaneously measuring TBW using HTO and TEBS using long-lived radiosodium. The values obtained with this method in 13 normal men are given.

METHODS

A solution containing 300 μc of HTO and 1.25 μc of Na^{22} Cl per milliliter was prepared in multiple dose ampules and sterilized by autoclaving. Two milliliters of the solution was administered intravenously with a syringe, calibrated to contain and not to deliver. The syringe was rinsed 3 times with the patient's blood after the injection and before removing the needle from the vein.

In 24 hours a 30 ml. specimen of blood was drawn; 10 ml. was discharged into a bottle containing balanced oxalate for HTO determination and the remainder was delivered into a test tube and permitted to clot for serum sodium determinations. A 24-hour urine sample was collected to permit estimation of urinary excretion of HTO and Na^{22}. The amount excreted in the urine was subtracted from the calibrated tracer dose of HTO and Na^{22} injected in estimating the total radioactivity in the body.

Tritium Assay.—Three milliliters of plasma, prepared from the oxalated blood, were pipetted into a 50 ml. flask, which was closed with a rubber stopper and frozen in a thin shell. The flask was attached to a collecting V-shaped tube, the angle of which was immersed in a mixture of solid CO_2 and isopropanol and connected to a vacuum manifold (Fig. 1). The water was transferred to the collecting tube by sublimation in a period of 2 to 3 hours.

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The collecting tube was removed, stoppered, and permitted to warm up to room temperature. Three milliliter samples of urine and of a diluted sample of the administered solution (diluted 1:1,000 for convenience in counting) were treated in an identical fashion.

Fig. 1.—Apparatus used for lyophilization of plasma and urine for estimation of TBW using HTO. *A*, Flask in which sample is frozen. *B*, V-shaped collecting tube which is partially immersed in solid CO_2-isopropanol mixture in Dewar flask (*C*). *D*, Manifold connected to vacuum pump through trap immersed in solid CO_2-isopropanol.

Duplicate 1 ml. samples of the water obtained from the plasma, urine, and the administered solution were measured into 2.5 ounce counting bottles* and 49 ml. of a scintillation solution† was added. The samples were counted, after cooling at least 4 hours, in a liquid scintillation counter at about 11° C. A blank containing 1 ml. of distilled water and 49 ml. of the scintillation solution was used for background determination. The TBW was calculated from the data obtained as follows:

$$\text{TBW in liters} = \frac{\text{total counts injected} - \text{Total counts in 24-hour urine}}{\text{counts per ml. of plasma water} \times 1{,}000}$$

Other Methods.—Radiosodium content of the injected solution, serum, and urine was estimated with a well scintillation counter and pulse height selector combination. The sodium concentration in serum was measured with a flame photometer. The TEBS was calculated from the radioactivity of the serum samples and the serum sodium concentration as follows:

$$\text{TEBS in equivalents} = \frac{\text{total counts injected} - \text{total counts in 24-hour urine}}{\dfrac{\text{counts per ml. serum} \times 1{,}000}{\text{Eq. of sodium per liter of serum}}}$$

The total body fat was estimated in the following manner. Skin fold thicknesses were measured in the midaxillary line at the level of the xiphoid (X_1), at the chest in the juxtanipple position (X_2), and on the dorsum of the arm at the midpoint between the tip

*Hazel-Atlas No. 3296, Crown Glass Co., Chicago, Ill.

†Three grams diphenyloxazole, 270 ml. absolute ethanol, 730 ml. toluene.

of the acromion and the tip of the olecranon (X_4), using calipers designed by the Army Medical Nutrition Laboratory.[4] The measurements were used to estimate body density (y) from the regression formula of Pascale, Grossman, and Sloane:[5]

$$y = 1.08012 - 0.007123X_1 - 0.00483X_2 - 0.005513X_4.$$

The per cent body fat (F) was then calculated by the equation of Keys and Brozek[6]:

$$F = \frac{4.201}{\text{Density}} - 3.813.$$

The estimated total body fat was then subtracted from the total body weight to obtain fat-free weight and permit calculation of TBW per kilogram of fat-free weight.

RESULTS

No detectable fractionation of HTO and H_2O occurred during the sublimation of water from plasma. To assess the extent of fractionation of HTO and H_2O during sublimation, a stock solution of HTO was prepared. A portion of the stock solution was used to reconstitute lyophilized nonradioactive serum and the resulting solution lyophilized. Aliquots of the water were obtained at approximately half-completion of the transfer and at the completion of sublimation. Statistically, comparison of the activities of these samples with samples of the original stock water solution by the "F test" revealed that there was no significant difference between the values obtained for the two groups. It is possible that water transfer occurs during lyophilization only from the surface of the frozen plasma, thus preventing differential vaporization of HTO and H_2O. No entrainment of radiosodium during sublimation of water from a sample of plasma containing an activity of radiosodium ten times that expected during the measurement of TEBS could be demonstrated by counting samples of the distillate in the well scintillation counter.

The counting jars used were remarkably uniform and, in testing a large number with a stock scintillation system, variation due to difference in the jars was less than 2 per cent of the count. More expensive thin-walled vials of 20 ml. capacity showed variations under the same conditions of as much as 8 per cent.

The variation in efficiency of the counting circuit, even when using the balance point technique of Arnold,[7] introduced considerable error in estimation of total body water. The low beta particle energy ($\bar{E}_\beta = 5.69$ Kev.) places high requirements on the photomultiplier and electronic circuits. Correction for change in efficiency was made by counting a sealed high activity standard tritium reference sample (containing approximately 15,000 counts per minute per ml.) between each unknown sample. The ratio of the average activity of the reference samples which bracket the unknown to a standard activity chosen for the reference sample served to correct for variations occurring during the time interval required for counting all the samples from a given study.

No significant change in the specific activity of the plasma water or serum sodium followed storage of samples in well-stoppered tubes in the frozen state, or of lyophilized samples of plasma water, for 7 days.

In order to estimate the error of measurement of tritium activity in plasma, HTO was added to a large sample of pooled plasma and 5 separate lyophilizations were carried out on samples from the pool. The water collected from each lyophilization was used to prepare two samples for counting. All activities were corrected for variation in efficiency of the counter. The average value obtained was 118,491 c.p.m. for each milliliter of plasma water, with a standard deviation of the individual estimations of ±1,781 and a coefficient of variation of 1.5 per cent.

Estimation of tritium activity in 5 samples of the same plasma pool, by the method of Langham and co-workers,[2] gave an average value of 88,910 c.p.m. per milliliter of plasma water, assuming that plasma is 92 per cent water, with a standard deviation of ±1,305 and a coefficient of variation of 1.6 per cent. The lower counting rate is the result of the smaller sample of plasma water counted and the lower efficiency of the Langham method.

To study the effect of plasma from patients with a variety of diseases on the activity of a standard quantity of tritium in the Langham technique, a sample of HTO was added to the 10 per cent TCA used in precipitating the protein. Since the same amount of the TCA solution was added to each sample, the same activity would be anticipated. The average values obtained in duplicate determinations on each sample are given in Table I.

TABLE I

DIAGNOSIS	AVERAGE C.P.M./ML. PLASMA WATER
Viral hepatitis (icteric plasma)	1,016
Nephrosis (lipemic and hypoproteinemic plasma)	993
Hemolytic anemia (icteric plasma)	969
Normal plasma	972

The coefficient of variation for this group of samples was 2.1 per cent, which is greater than that observed for replicates of a single sample of plasma. This suggests that differences in plasmas contribute to error in estimation of HTO, although the greater coefficient of variation may be attributable in part to the error introduced by an additional pipetting of TCA containing the tritium.

Previous work has shown that the apparent size of the exchangeable body sodium pool becomes fairly constant 18 hours after injection of a tracer dose of radiosodium.[8, 9] Subsequent exchange takes place at an extremely slow rate, making it possible to use a single sample of blood taken at 24 hours to estimate TEBS.

The validity of using the results obtained from a single sample of blood drawn 24 hours following injection of tritium to estimate TBW along with TEBS was tested in 9 patients with minor illnesses. Samples of blood were obtained at 3, 5, 7, and 24 hours for estimation of the TBW. The apparent TBW calculated at each sample period was plotted against time, using semilog paper. The points fell on a straight line which was extrapolated to obtain

an apparent value for TBW at zero time. Two examples are shown in Fig. 2. For the 9 patients, the average apparent increase in TBW over the extrapolated value was 0.7 per cent at 3 hours, 1.6 per cent at 5 hours, 2.5 per cent at 7 hours, and 3.9 per cent at 24 hours. Excretion of HTO in the urine, for which a correction was not made in this series, would account for most of the apparent increase. (For 13 normal subjects, reported below, the average 24-hour urinary excretion was 2.9 ± 1.5 per cent of the injected dose of tritium.)

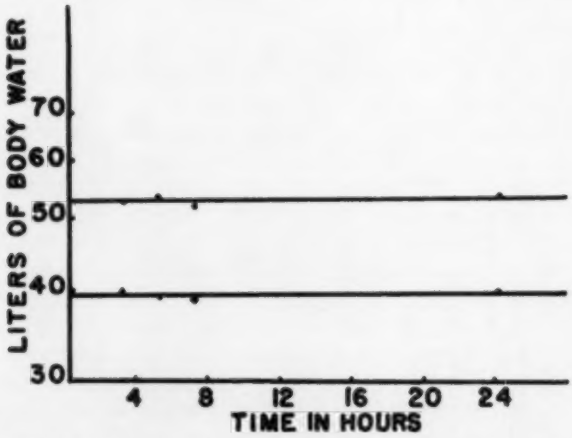

Fig. 2.—Calculated TBW as a function of time in hours after administration of a tracer dose of HTO.

The reproducibility of estimating TBW and TEBS in the same individual was established by repeating the injection of tracer doses on successive days in two subjects. The radioactivity of the sample of plasma obtained 24 hours after the first injection furnished the base-line values for the second determination. The values obtained are shown in Table II. The largest variation in TBW was 1.2 per cent which is within the error of the tritium estimation. The variation in TEBS was larger (5.9 per cent), but this represents only 2.8 Gm. of sodium out of an exchangeable pool of about 24 Gm.

TABLE II

SUBJECT	BODY WATER (LITERS)		EXCHANGEABLE Na (EQUIV.)	
	DAY 1	DAY 2	DAY 1	DAY 2
T. U.	50.16	50.76	3.36	3.16
S. O.	39.26	39.33	2.46	2.58

To establish normal values for TBW and TEBS using the methods described, studies were carried out on 13 normal male volunteers whose ages ranged from 23 to 49 years. The data are given in Table III. The heights varied from 158.8 cm. to 185.4 cm. and the body weights from 57.1 to 98.6

TABLE III. NORMAL VALUES

SUBJECT	AGE	WEIGHT (KG.)	HEIGHT (C.M.)	BODY FAT* (PER CENT)	BODY WATER (LITERS)	MILLILITERS OF BODY WATERS PER KILOGRAM BODY WEIGHT	MILLILITERS OF BODY WATER PER KILOGRAM LEAN BODY WEIGHT	EQUIVALENTS OF SODIUM	MILLIEQUIVALENTS OF SODIUM PER KILOGRAM BODY WEIGHT	MILLIEQUIVALENTS OF SODIUM PER KILOGRAM FAT-FREE BODY WEIGHT	MILLIEQUIVALENTS OF SODIUM PER LITER BODY WATER
E. T.	33	69.4	158.8	13.29	42.62	614	708	3.05	43.9	50.7	71.5
R. S.	49	57.1	177.2	11.66	39.26	688	779	2.46	43.1	48.8	62.7
A. F.	47	58.3	172.7	10.42	39.50	678	757	2.69	46.1	51.5	68.2
P. W.	41	88.9	179.1	13.68	55.18	621	720	3.47	39.1	45.3	62.9
G. G.	27	76.4	170.2	20.27	42.04	550	690	2.77	36.2	45.5	65.9
D. S.	23	65.1	182.2	12.77	42.85	658	754	3.04	46.7	53.5	70.9
F. V.	26	82.7	179.1	20.39	48.18	583	732	3.17	38.3	48.2	65.8
C. K.	23	98.6	182.9	20.50	55.95	567	714	3.43	34.8	43.7	61.3
J. L.	25	63.2	171.5	14.41	38.31	601	708	2.56	40.5	47.3	66.8
C. L.	24	90.9	185.4	16.35	57.17	629	752	3.52	38.7	46.3	61.6
S. H.	38	63.1	166.4	15.83	37.50	596	706	2.92	46.3	55.0	77.9
D. R.	30	88.9	182.9	21.38	51.04	574	730	3.35	37.7	47.9	65.6
J. V.	45	89.3	175.3	18.48	47.33	530	646	3.12	34.9	42.8	65.9
Mean		76.3	175.7	16.11	45.92	607	723	3.04	40.5	48.2	66.7
Standard deviation		–	–	±3.75	±7.05	±47.91	±34.24	±0.35	±4.30	±3.67	±4.64

*Based on skin fold thickness.

Kg. Total body fat, estimated from skin fold thickness measurements, varied between 10.4 and 21.4 per cent of the total body weight. The TBW ranged between 37.50 and 57.17 liters, and the TEBS varied between 2.46 and 3.52 equivalents. The average TBW was 607 ± 47.9 ml. per kilogram of body weight and 723 ± 34.2 ml. per kilogram of fat-free body weight. The average TEBS was 40.5 ± 4.3 mEq. per kilogram of total body weight, 48.2 ± 3.67 mEq. per kilogram of lean body weight, and 66.7 ± 4.64 mEq. per liter of TBW. The average excretion of radiosodium in the urine for the 24-hour period was 4.51 ± 1.6 per cent of the administered dose with a range of 2.3 to 7.7

DISCUSSION

The method for measuring TBW using HTO appears to have acceptable accuracy using a tracer dose of 600 μc. The sublimation procedure is simple, rapid, and the apparatus used can accommodate a large number of samples. The preparation of a pure sample of water from plasma or urine permits direct calculation of the tritium activity per milliliter of water without assumptions about the water content of these fluids. In addition, it permits the accurate estimation of tritium in the presence of other radioisotopes.

The control of variables which introduce errors in the estimation of tritium by liquid scintillation counting is necessary to achieve the accuracy desired in TBW determinations. The method presented appears to have several advantages over the previously described technique of Langham and co-workers: it gives a higher counting rate per microcurie of HTO present in the plasma, permitting smaller tracer doses to be used; it allows TBW to be measured with HTO in the presence of other nonvolatile radioisotopes present in plasma; the results are not influenced by variations in protein content, fat content, or pigments in the plasma (Table I).

The estimation of TBW from the results of a single 24-hour sample was found to be reasonably accurate. Even without correcting for urinary excretion, it would introduce an error of less than 25 ml. per kilogram as compared to an extrapolated value based on the results of multiple samples. (To minimize this source of error in establishing normal values, correction for urinary excretion of HTO and radiosodium was made.) Reproducibility for combined determinations of TBW and TEBS was established on successive days in two instances.

The values for TBW for the group of normal young men studied agree with those given in the literature based on isotope dilution methods[10-12] and agree fairly well with some based on antipyrine methods.[12-14] They are higher than those reported by others using tritium,[15] deuterium,[16] and antipyrine.[16-20] Differences in sex, nutritional status, and age distribution of the subjects, which have been shown to effect total body water content, may account in part for such discrepancies.[10, 11, 21, 22] In addition, the antipyrine method gives lower values than deuterium or tritium in general because of plasma binding of the antipyrine[17] and the small exchange of hydrogen isotopes with body constituents.[15]

The dispersion within the group is less for values of TBW per kilogram of fat-free body weight than those based on uncorrected body weights. The average value for normal subjects of 723 ml. of water per kilogram of fat-free body weight corresponds well with estimates that the total body water is about 72 per cent of the fat-free body weight.[1, 6, 22] This close agreement lends validity to the estimation of body fat from skin fold thickness in normal adult men. Calculation of body fat from the TBW, as a proportion of total body weight using the formula of Keys and Brozek[6] gave an average value for the group of 14 per cent. This agrees well with the value of 16 per cent based on skin fold thickness measurements. It has been pointed out by others that these formulations for estimating body fat probably do not apply when a rapid change in body weight or in the state of hydration has occurred.[1, 6]

The values obtained in the present study for TBS agree closely with those previously reported.[8, 9] The variation in values for TEBS per kilogram of lean body weight is smaller than the value calculated on the basis of total body weight. This is probably due to the low content of sodium in adipose tissue.[22] If TEBS is expressed as milliequivalents per liter of TBW, as suggested by Ikkos[16] and later by Farber,[20] an average value of 66.7 ± 4.6 is obtained. This value is lower than that of 74.7 mEq. of Na per liter of TBW obtained by Ikkos and that of 81.3 obtained by Farber. In the former report the values for both TBW and TEBS are lower and in the latter the discrepancy exists only in a lower value for TBW.

SUMMARY

1. Methods have been devised for simultaneous measurement of total body water (TBW) and total exchangeable body sodium (TEBS), using tritium and radiosodium, respectively. Sublimation of the water from frozen plasma or urine makes it possible to measure HTO in the presence of other isotopes and avoid errors introduced by differences in protein, lipid, and pigment content of plasma or urine.

2. For accurate estimation of HTO by liquid scintillation counting, temperature of the samples, uniformity of counting bottles, and variations in efficiency of the counting system must be carefully controlled.

3. In 13 normal men the TBW varied between 37.5 and 57.2 liters. The average TBW was 61 per cent of the total body weight and 72 per cent of the fat-free body weight. The average TEBS was 40.5 ± 4.3 mEq. per kilogram of total body weight, 48.2 ± 3.7 mEq. per kilogram of lean body weight, and 66.7 ± 4.64 mEq. per liter TBW.

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A REVERSIBLE EFFECT ON THE METABOLISM OF HUMAN ERYTHROCYTES BY *p*-CHLOROMERCURIBENZOIC ACID AND *N*-ETHYL MALEIMIDE

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SULFHYDRYL groups are necessary to maintain the integrity of normal human erythrocytes. If these groups are neutralized by sulfhydryl reagents such as phenylmercuric chloride[1] or *p*-chloromercuribenzoic acid,[2] the cells hemolyze in a predictable manner. *N*-Ethyl maleimide does not cause hemolysis[3] of normal human erythrocytes although it is capable of reacting with the sulfhydryl groups of hemolyzed cells. This led us to speculate that the intact cell membrane might prevent access of *N*-ethyl maleimide to the sulfhydryl groups of the cells. These studies were designed to observe the effect of *N*-ethyl maleimide and *p*-chloromercuribenzoic acid on another parameter of cell function.

METHODS

Preparation of Erythrocytes.—Fresh human blood was placed in a tube that contained 1 mg. of heparin* per 5 ml. of whole blood. After centrifugation the plasma, leukocytes and platelets were discarded. The erythrocytes were washed 3 times in cool 0.9 per cent saline. After the final washing, the cells were left packed and added to a 0.9 per cent sodium chloride solution to make a 40 per cent suspension. The suspension was rotated gently in an Erlenmeyer flask for 10 minutes to permit complete oxygenation of the hemoglobin.

Manometric Method.—Three milliliters of an oxygenated suspension of human erythrocytes was pipetted into each Warburg vessel.[4] Two-tenths milliliter of 20 per cent KOH was placed in the center well. One milliliter of the appropriate concentration of sulfhydryl inhibitor or glutathione was placed in the side arm of the flask. The vessel was then attached to a Warburg manometer in the usual manner and immersed in a water bath maintained at 37° ± 0.5° C. The manometers were shaken at a rate of 120 strokes per minute. The stopcocks were left open for 15 minutes to permit equilibration, then they were closed and the period of observation began.

Reagents.—*N*-Ethyl maleimide (NEM) was purchased from Schwarz Laboratories, the sodium salt of *p*-chloromercuribenzoic acid (PCMB) from Sigma Chemical Co., and glutathione (GSH) from the Nutritional Biochemical Corporation. Solutions of the appropriate reagents were made fresh daily. A 0.9 per cent solution of sodium chloride was used as the solvent. When possible, the amount of reagent in a flask was varied in each experiment by appropriate dilution of the day's stock solution with 0.9 per cent sodium chloride solution; this decreased the variation from errors in weighing which would occur if each increment was weighed individually.

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RESULTS

Experiment 1.—The oxygen uptake of fresh normal human erythrocytes (Fig. 1) was measured for 60 minutes. As soon as the 60-minute reading was recorded, NEM in increasing increments was rinsed from the side arm into the flask. In each side arm, NEM was dissolved in 1 ml. of saline in the following amounts: 1, 3, 5, 7, 9, and 11 mg. Subsequent oxygen uptake was abolished except for a slight increase in the manometer containing 1 mg. of NEM. There was slight hemolysis in the flask with 11 mg. of NEM; the others had none.

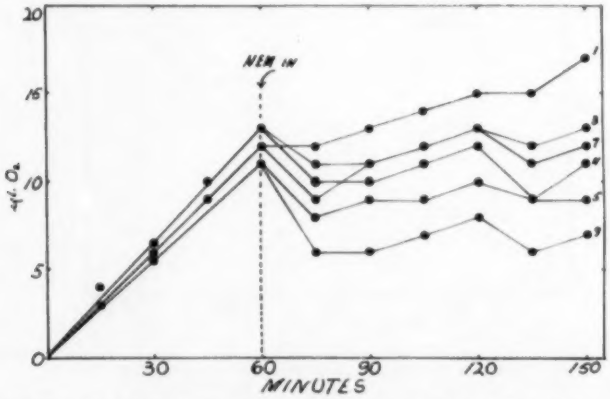

Fig. 1.—The effect of *N*-ethyl maleimide on the uptake of 3 ml. of a 40 per cent suspension of washed erythrocytes in 0.9 per cent sodium chloride solution. The number of milligrams of NEM tipped in from the side arm at 60 minutes is shown with the curves.

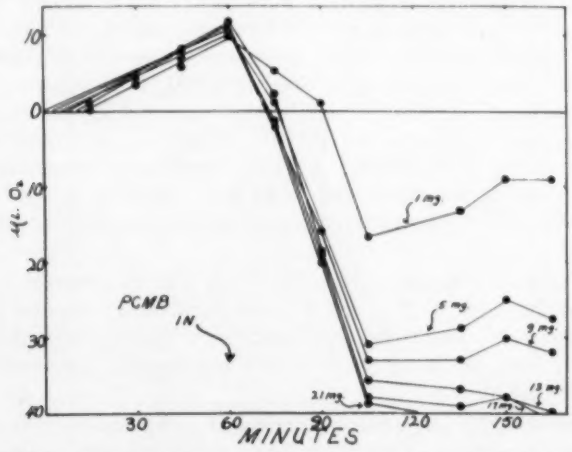

Fig. 2.—The effect of p-chloromercuribenzoic acid on the oxygen uptake of 3 ml. of a 40 per cent suspension of erythrocytes. There was striking evolution of oxygen when the cells were treated with PCMB.

Experiment 2.—PCMB was substituted in this experiment (Fig. 2) for NEM as used in Experiment 1. The PCMB was dissolved in 1 ml. of saline

in the side arm in the following amounts: 1, 5, 9, 13, 17, and 21 mg. Hemolysis was complete in all flasks except those with 1 mg. and 5 mg. where it was incomplete. During the 75 minutes after tipping in the PCMB, a gas evolved that was not removed by the KOH in the center well. The amount of oxygen evolved was greater with higher concentration of the PCMB.

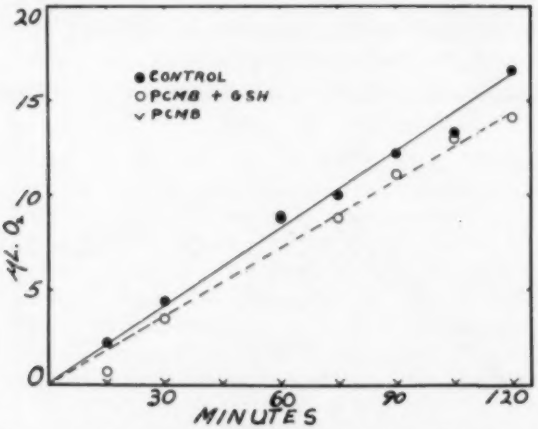

Fig. 3.—PCMB stopped the oxygen uptake of erythrocytes. This action was prevented if PCMB was previously neutralized with glutathione.

Experiment 3.—A. In this study (Fig. 3) the variations were performed in duplicate and the curves plotted as the average. The control pair of flasks contained only erythrocytes in saline. Five milligrams of PCMB was added to the erythrocyte suspension in each of two other flasks. In the third pair, 5 mg. of PCMB which had reacted with an equivalent amount of glutathione for 10 minutes was added. Equilibration was permitted for 15 minutes before the stopcocks were closed. Similar oxygen uptake occurred in the control flasks and in those containing neutralized PCMB. There was no oxygen uptake in the flasks containing only PCMB. *B.* This experiment was similar to 3,A except that 5 mg. NEM was substituted for PCMB. Again an equivalent amount of GSH was used. NEM prevented oxygen uptake but this effect was neutralized by GSH.

Experiment 4.—NEM was added (Fig. 4) to a saline suspension of erythrocytes in amounts of 5, 3, 2, 1, and 0.5 mg. per flask 15 minutes before the stopcocks were closed. The highest concentration of NEM inhibited all oxygen uptake. In the other instances the uptake was indirectly proportional to the concentration of NEM.

Experiment 5.—This experimental system (Fig. 5) was composed of 3 ml. of a 40 per cent suspension of erythrocytes in saline and varying quantities of GSH. The respective amounts of GSH, 6, 9, 12, 24.6, and 36.9 mg., were dissolved in 1 ml. of 0.9 per cent sodium chloride solution and added to the cells 15 minutes before the stopcocks were closed. Each was performed in duplicate.

The uptake of oxygen was greater in all flasks than in the control except for the pair containing 36.9 mg. per flask. The greatest increase occurred with 12 mg. GSH. The uptake of the pair with 24.6 mg. of GSH was greater than the control but less than the pair containing 6 mg. of GSH. An unusual amount of hemolysis occurred in those flasks containing 24.6 and 36.9 mg. of GSH, 4.5 and 6.4 per cent, respectively.

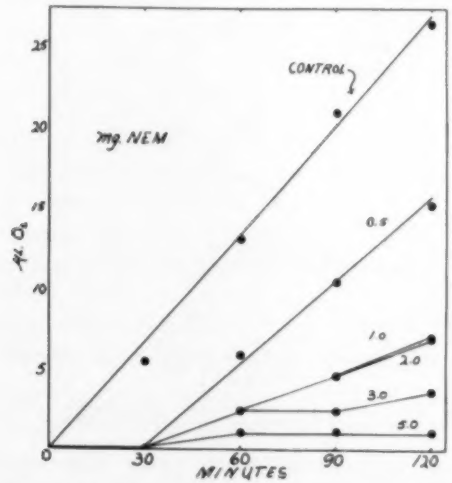

Fig. 4.—NEM in amounts designated on the curves decreased the oxygen uptake of red cells.

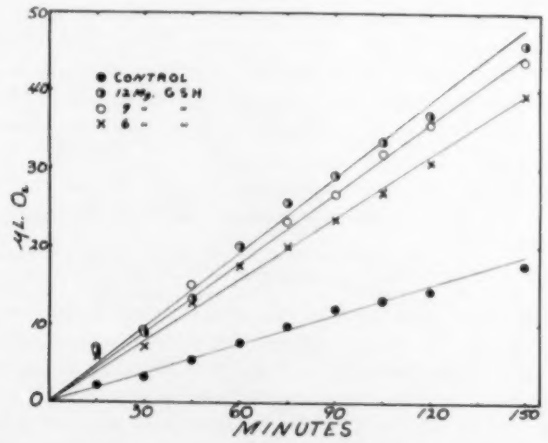

Fig. 5.—Glutathione increased the oxygen uptake of human erythrocytes.

Experiment 6.—Since NEM effectively blocks the uptake of oxygen by erythrocytes without causing hemolysis, this experiment (Fig. 6) was designed

to determine if GSH would reinstitute oxygen uptake of cells. Three milliliters of a 40 per cent suspension of erythrocytes was placed in each flask. NEM was added directly in 0.5 ml. saline, and 0.5 ml. (12.3 mg.) GSH was placed in the side arm so that it could be tipped at intervals.

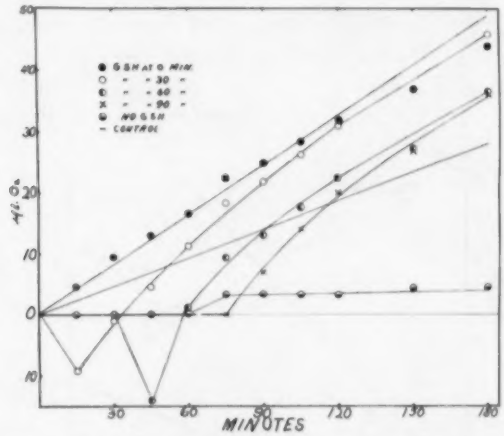

Fig. 6.—Human red cells were first treated with NEM. The addition of glutathione at intervals as long as 90 minutes re-established the oxygen uptake in each instance.

The untreated cells had a normal oxygen uptake. The erythrocytes treated with NEM only had no oxygen uptake. Those with GSH only had an uptake nearly twice normal. In the other 3 flasks the cells were incubated in NEM for 30, 60, and 90 minutes before GSH was added. The first reading following the addition of GSH showed evolution of a gas in the 30- and 60-minute flasks but none in the 90-minute one. In all instances the oxygen uptake recovered and proceeded at accelerated rates. GSH was present in excess in these studies. In other studies where an equimolar amount of GSH was added, the accelerated uptake did not occur.

The oxygen uptake of erythrocytes treated with PCMB was reactivated with an excess of GSH in a similar manner, although hemolysis interfered after sufficient time.[2]

DISCUSSION

These studies show that both of these sulfhydryl reagents inhibit the oxidative mechanism of the human erthrocyte but only *p*-chloromercuribenzoic acid disrupts its cellular integrity. *N*-Ethyl maleimide is as effective as *p*-chloromercuribenzoic acid in abolishing the uptake of oxygen yet it does not cause hemolysis. It is not clear at the present time whether this metabolic effect is mediated through the glutathione reductase,[8] or some other enzyme system. Bruns and Rummel[5] found that glutathione and cysteine increased the oxygen consumption and carbon dioxide production of erythrocytes. They implicated the hexosemonophosphate system of Warburg and Christian.

A difference in permeability of the cell to *p*-chloromercuribenzoic acid and *N*-ethyl maleimide might explain the different hemolytic effect but not the similar metabolic effect. LeFevre[6] reported that the permeability of human erythrocytes to nonelectrolytes was increased by *p*-chloromercuribenzoic acid as well as some other enzyme poisons. No alteration in radioactive phosphorus exchange was caused by glutathione and Mg. ++ in the studies of Prankerd and Altman.[7]

At present, the effect of *N*-ethyl maleimide appears to offer interesting possibilities for further study because it inhibits a metabolic function of the cells without disrupting their integrity.

SUMMARY

1. Both *p*-chloromercuribenzoic acid and *N*-ethyl maleimide stopped the oxygen uptake of human erythrocytes.

2. In each instance, glutathione reversed the effect.

3. In certain concentrations, glutathione increased the oxygen uptake of human erythrocytes.

4. Although both *N*-ethyl maleimide and *p*-chloromercuribenzoic acid stop the oxygen uptake of human erthrocytes, only the latter caused hemolysis as well.

We wish to acknowledge the technical assistance of Rodney L. Bidlack, Richard L. King, and Helen E. Lemme.

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LABORATORY METHODS

A SCREENING TEST FOR THE HEMAGGLUTINATION REACTION IN RHEUMATOID ARTHRITIS

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A SIMPLE screening test which can be done before the quantitative hemagglutination reaction in rheumatoid arthritis is carried out is described below. Information in detail about the application of human type O erythrocytes is also presented.

Wager[1] was the first who found that human cells sensitized with the homologous rabbit amboceptor *in principe* can be used for the hemagglutination reaction in rheumatoid arthritis. For some years in our laboratory human type O cells have been successfully used in this test,[2] and others have recently recommended their use.[3] The greater instability of the human erythrocytes, as compared with sheep cells, presented a difficulty which was overcome by the use of blood preserved in a glycerol-citrate mixture in a Deepfreeze cabinet.[4] The use of human type O cells has the obvious advantage of eliminating the need for absorbtion of the sera with nonsensitized cells.

MATERIALS AND METHODS

Human type O blood is collected in an equal volume of modified Alsever's solution.

A preserving fluid containing one volume of glycerol and one volume of 6 per cent trisodium citrate (dihydric) gave the best results when a Deepfreeze cabinet at -40° C. was used.

Washing Fluids.—Fluids A, B, and C were used containing, respectively, 16, 8, and 4 per cent of glycerol (w/v) in 3 per cent trisodium citrate. Solution A is obtained by mixing 65 ml. of glycerol, 185 ml. of distilled water and 250 ml. of 6 per cent trisodium citrate. Solution B is made by mixing 32.5 ml. of glycerol, 217.5 ml. of distilled water, and 250 ml. of 6 per cent trisodium citrate; for solution C mix 16.25 ml. of glycerol, 233.75 ml. of distilled water, and 250 ml. of 6 per cent trisodium citrate.

The mixture of human blood and Alsever's solution is kept 1 to 3 days at + 4° C. Then it is brought to room temperature and the supernatant fluid is removed and discarded by suction and centrifuging. After measuring the volume of erythrocytes, an equal volume of the preserving fluid is added slowly, agitating the mixture continuously. Then the mixture is divided into small tubes in 3 ml. portions and placed at -40° C., where it can be kept for several months. Before use the mixture is thawed and centrifuged. Then the cells are washed 3 times using washing fluids A, B, and C, respectively, followed by 3 washings in saline. The

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sensitized suspension is prepared by adding one volume of a suitable dilution of amboceptor (see below) to one volume of 1 per cent erythrocyte suspension and allowing the mixture to stand for 15 minutes at 37° C. in a water bath.

Amboceptor.—Rabbits are injected intravenously 8 to 10 times with a 5 per cent human type O erythrocyte suspension, giving 2 doses a week: the first week 0.5 ml. and 1 ml. and thereafter 2 ml. doses. The rabbit blood is collected one week after the last injection. Fewer injections yielded unsuitable amboceptors. The sensitizing amboceptor dilution is experimentally determined by titrating some positive and some negative sera with cells sensitized with different dilutions of the amboceptor. In our hands the optimal concentration appeared to lie between ¼ and ⅙ of the minimum agglutinating dose. This dose is controlled monthly. With our amboceptor it appeared to be unnecessary to inactivate the complement in the patient's sera. Lysis within the given amboceptor dilutions was not observed. Omission of the inactivation step proved to be an advantage as fewer nonspecific reactions in low titer were now found in sera from healthy persons. Only 4 of 124 (3.2 per cent) of sera from healthy persons were found to have a titer of 1:8; higher titers were not seen, with the single exception of one serum with a titer 1:128.

Screening Test.—Slides are divided into 3 parts by paraffin strokes. One drop of patient's serum diluted 1:8 is placed on the slide and mixed with one drop of the sensitized cell suspension, thus giving a final serum dilution of 1:16. After 5 minutes of tilting, the result is macroscopically read and expressed as follows: − = no agglutination; ± = weak agglutination; + = agglutination; ++ = strong agglutination.

Quantitative Test.—The sera are serially diluted in saline in 0.5 ml. volumes (1:4, 1:8, 1:16, etc). To each tube is added 0.5 ml. sensitized cells, the final serum dilutions thus becoming 1:8, 1:16, 1:32, etc. A control tube is added containing 0.5 ml. serum 1:4 and 0.5 ml. 0.5 per cent nonsensitized cells. A positive serum of known titer is titrated with each group of sera to be investigated. The tubes are incubated in a water bath at 37° C. for 2 hours and placed in the refrigerator overnight. A titer of 1:8 is considered as doubtful, 1:16 and higher as positive.

RESULTS

Eight-hundred and four sera received for serologic investigation for rheumatic diseases were tested using the slide test as well as the quantitative test. The results are given in Table I.

TABLE I. COMPARISON OF RESULTS OBTAINED WITH SLIDE TEST AND QUANTITATIVE TEST

NUMBER OF SERA	SLIDE TEST	QUANTITATIVE TEST
550	−	—
10	−	1:8
24	±	—
37	±	1:8 to 1:16
14	±	1:32 to 1:128
8	+	—
62	+	1:8 to 1:256
2	++	1:16
97	++	1:32 to 1:4,096

Of 560 sera negative by the slide test, 10 had a titer of 1:8 in the quantitative test. As this number is small and the titer 1:8 is of doubtful value, no great mistake is made when these sera are considered negative. The screening test thus answers its purpose in that only the sera positive by the slide test have to be titrated. However, we have been somewhat more rigorous and have proceeded as follows: sera negative in the slide test are titrated in the dilutions 1:8 and 1:16 only, sera ± and + are titrated in the dilutions 1:8 to 1:256, sera ++ in the slide test are titrated in the dilutions 1:8 to 1:4,096.

DISCUSSION

The advantage of a screening test for the hemagglutination reaction in rheumatoid arthritis is obvious. The test would be most useful when only sera positive in the slide test needed to be titrated. From 560 sera negative in the slide test, 10 had a titer 1:8 in the quantitative test, a number which is negligible when the doubtful significance of such a titer is taken into consideration. Undiluted serum appeared to be unsuitable for the screening test as with it many doubtful positive reactions were obtained with sera from healthy persons and, on the other hand, some positive reactions were missed.

We have used type O Rh positive cells. With these, there is the added possibility of agglutination by anti-Rh agglutinins as well as by alpha-2 agglutinins. Such a reaction would be indicated as being a false positive by the unsensitized cell control test in the later quantitative reaction. In our material, however, we have not seen such positive controls.

A screening test for rheumatoid arthritis sera with latex polystyrene particles coated with gamma globulin has been described by Rheins and associates.[5] This test is somewhat more complicated than ours as incubating at 37° C. or 56° C. for half an hour and centrifugation are necessary. On the other hand, the reagents for the latex test are easy to prepare. It will be of interest to compare the clinical value of these two methods.

SUMMARY

A slide test employing human type O erythrocytes has been found useful as a simple screening test for hemagglutination in the sera of patients with rheumatoid arthritis.

We are indebted to Miss T. Smit for valuable technical assistance.

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THE QUANTITATIVE DETERMINATION OF DRUG-RESISTANT BACTERIA BY INDUCED ENZYME SYNTHESIS

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PRESENT procedures for testing the susceptibility of microorganisms to chemotherapeutic agents are based on the inhibition of bacterial growth. Reports that many antimetabolic agents inhibit protein synthesis, and therefore block the synthesis of induced (adapted) enzymes, suggest that a method based on induced enzyme synthesis can be devised for the quantitative determination of drug-resistant organisms in the presence of drug-sensitive organisms.

Creaser[1] observed that β-D-galactosidase formation by *Micrococcus pyogenes* var. *aureus* is inhibited by a number of antimetabolic agents including streptomycin, dihydrostreptomycin, Chloromycetin and Aureomycin. Kushner[2] has demonstrated that chloramphenicol inhibits induced enzyme synthesis in chloramphenicol-sensitive strains of *Pseudomonas fluorescens,* but enzyme synthesis is not inhibited in chloramphenicol-resistant strains of the organism. The formation of β-D-galactosidase is inhibited by dihydrostreptomycin in dihydrostreptomycin-sensitive strains of *Escherichia coli,* but not in strains resistant to the antibiotic.[3]

A test system based on adaptive enzyme synthesis would have the advantage that the adaptive enzyme is detected against a background devoid of this enzymatic activity. Since the drug-sensitive cells will not form the adaptive enzyme in the presence of the drug, a relatively small number of drug-resistant organisms can be detected in the presence of a large number of the sensitive cells. When the test system is based on growth, overinoculation of the medium can obscure the true ratio of drug-resistant to sensitive organisms.

A further advantage of such a method lies in the inherent sensitivity of enzyme techniques which, when properly applied, may offer a means of detecting bacterial growth considerably earlier than is possible by direct inspection of the culture medium.

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Since a large body of published information exists on the induced β-D-galactosidase of *Esch. coli*, this enzyme was chosen for study.

MATERIALS AND METHODS

Medium and Microorganisms.—The "C" medium of Roberts and associates[4] was used throughout this work. Drug medium was the "C" medium to which streptomycin* (SM) was added to a final concentration of 100 μg per milliliter after the medium had been autoclaved. *Esch. coli*, strain K12 (W-1485), streptomycin-sensitive (SM^s) and its streptomycin-resistant (SM^k) variant (W-3001) were obtained from Dr. Joshua Lederberg of the Department of Genetics, University of Wisconsin.

Orthonitrophenyl-β-D-galactoside: In the early phase of this work generous amounts of orthonitrophenyl-β-D-galactoside (ONPG) were supplied by Dr. Joshua Lederberg. ONPG was subsequently obtained from Quimotecnica Chilena, Ltd.

Plan of experiments: Lactose was used both as inducer of the galactosidase and as carbon source for the induced cells. Cells capable of undergoing induction were inoculated into medium containing lactose. Induction was allowed to proceed; an increase in the number of cells then took place. At various times following inoculation of the sample into the test medium aliquots were removed for enzyme assay.

Preparation of samples for assay: Twenty milliliter samples of cultures which had undergone induction were centrifuged at 20,000 × g in the International PR-2 refrigerated centrifuge for 20 minutes. The pellet was suspended in medium without the energy source and diluted to 2.0 ml. in microvolumetric vials; the optical density was read at 420 mμ in the Beckman Model DU spectrophotometer, and the dry weight bacterial cells per milliliter was estimated from a standard curve correlating optical density and dry weight.

Extraction of the enzyme and assay of β-D-galactosidase: The enzyme was released by incubating the 2.0 ml. cell suspension with 2 drops of toluene at 38° C. for one hour. Suspensions may be stored at 4° C. for at least 48 hours without a loss in enzyme activity. A modification of the method of Lederberg[5] was used to assay the enzyme. The assay mixture contained the following: 1.5 ml. buffer of pH 7.2 (0.2 M NaCl; 0.08 M Na_2PO_4), 0.3 ml. 0.005 M ONPG, and crude enzyme preparation (0.03 to 0.20 ml.); the final volume was 2.7 ml. The reaction was allowed to proceed for 20 minutes at 38° C. Addition of 0.3 ml. 1 M K_2CO_3 stops the reaction and intensifies the color of the orthonitrophenol (ONP) released by the hydrolysis of ONPG. One unit of enzyme is defined as that amount which catalyzes the hydrolysis of 1.0 μM ONPG per minute under the assay conditions. Specific activity of the enzyme is expressed as units of enzyme activity per milligram dry weight bacterial cells. The molecular extinction coefficient of ONP in 0.1 M K_2CO_3 at 420 mμ is $4.43 \pm 0.09 \times 10^6$ cm.^{2} × mole^{-1}.

RESULTS

The Effect of the Number of Viable Cells in the Inoculum on Induced Enzyme Synthesis and Growth.—Medium containing lactose (0.5 per cent) as the energy source was inoculated with a known number of unadapted SM^s cells. Inoculum size was estimated from a standard curve correlating optical density at 420 mμ and the number of viable cells per milliliter; the actual inoculum size was determined by plate count. The inoculated flasks were incubated at 37° C. on a shaking machine and sampling for the galactosidase assay was begun at a suitable time after inoculation. Fig. 1 shows that the intervals between inoculation and the appearance of measurable amounts of both the induced

*Streptomycin sulfate, E. R. Squibb & Son, New York, N. Y.

enzyme and bacterial growth are functions of the number of viable cells in the inoculum. Using the SM^R variant, results identical with those above were obtained both in drug-free and drug-containing medium.

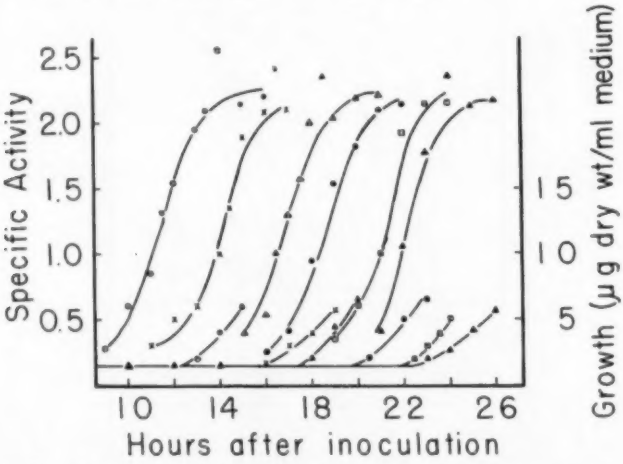

Fig. 1.—The effect of the number of viable cells in the inoculum on the length of the time periods between inoculation and the production of measurable amounts of induced enzyme (upper curves) and cells (lower curves). O—O = 3.2×10^3 cells per milliliter; x—x = 1.0×10^3 cells per milliliter; Δ—Δ = 2.9×10^2 cells per milliliter; ●—● = 1.1×10^2 cells per milliliter; □—□ = 5.8×10^1 cells per milliliter; ▲—▲ = 2.8×10^1 cells per milliliter.

The Rate of Induced Enzyme Synthesis and Growth of Mixed Cultures of Resistant and Sensitive Cells.—Mixed cultures were prepared by combining known numbers of unadapted sensitive and resistant cells; plate counts were made on drug-free and drug-containing media to determine the actual inoculum size. Drug-free and drug-containing media containing lactose (0.5 per cent) as the energy source were inoculated with a dilution of the mixed culture giving the desired concentration of cells per milliliter. The remainder of the procedure was as previously described. Fig. 2 shows the results of 4 experiments in which the mixed culture inocula were approximately 100 cells per milliliter; the variable is the number of resistant cells per milliliter. The first curve is the average rate of enzyme synthesis in drug-free medium in all experiments and the other curves show the times required for synthesis of β-D-galactosidase by mixed cultures in drug-containing medium. It may be seen that the intervals between inoculation and the detection of measurable amounts of both adaptive enzyme and bacterial growth in medium containing streptomycin is a function of the number of cells that are viable in the presence of the drug.

To avoid the difficulties inherent in measuring the time required to reach very low and very high specific activities of the galactosidase activity, levels of 0.50 and 1.50 were arbitrarily selected as standards. Similarly, arbitrary values for growth of 0.0020 and 0.0050 mg. dry weight bacterial cells per milliliter were selected as standards. Fig. 3 shows the straight-line relationship between the log of the inoculum size and the time required for the induced galactosidase to reach either of these standard activities. Consequently, if the time

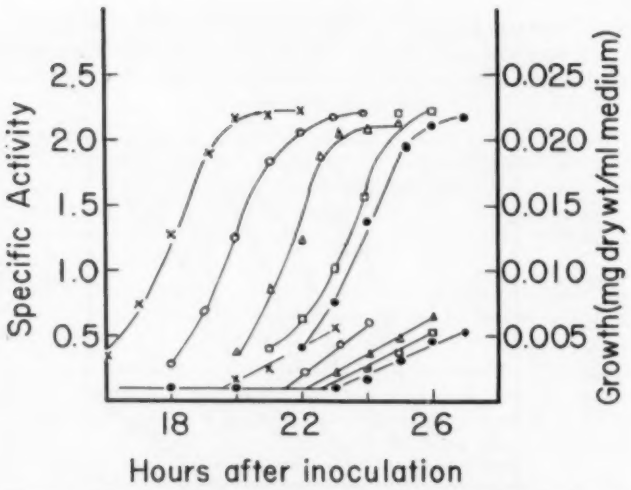

Fig. 2.—Same as Fig. 1 except that mixed cultures containing both SM^R and SM^S cells were inoculated into drug-free and drug-containing media. x—x = growth and induced enzyme formation of 1.16×10^2 cells per milliliter in drug-free medium (average of 4 experiments); O—O = 1.4×10^2 cells per milliliter (36 per cent SM^R, 64 per cent SM^S); △—△ = 1.06×10^2 cells per milliliter (35 per cent SM^R, 65 per cent SM^S); □—□ = 1.00×10^2 cells per milliliter (22 per cent SM^R, 78 per cent SM^S); ●—● = 1.15×10^2 cells per milliliter (11 per cent SM^R, 89 per cent SM^S). The last 4 curves represent induced enzyme synthesis and growth in drug-containing medium (100 μg SM per milliliter).

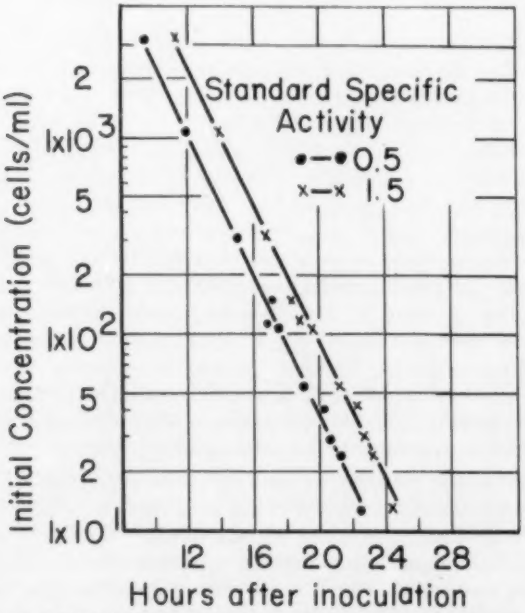

Fig. 3.—Relation between the initial concentration of viable cells in the inoculum and the time required for the enzyme to reach arbitrary standard specific activities of 0.50 and 1.50.

needed to reach either of these activity levels is known the number of viable cells in the inoculum can be estimated very closely. Fig. 4 shows that the time after inoculation required to reach these standard levels of growth is again a function of inoculum size.

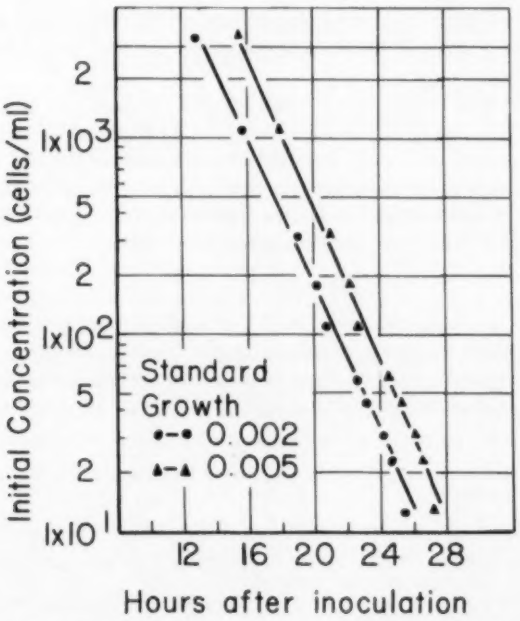

Fig. 4.—Relation between the initial concentration of viable cells in the inoculum and the time required to reach arbitrarily chosen standard growths of 0.0020 and 0.0050 mg. dry weight cells per milliliter of medium.

DISCUSSION

The above results demonstrate that a drug-susceptibility test based on adaptive enzyme synthesis may be completed in a shorter time than one based on currently available methods. Present methods for the detection of drug-resistant organisms in the presence of drug-sensitive organisms are based on growth. A problem inherent in these methods is overinoculation. If the sample used as inoculum contains a large absolute number of drug-resistant organisms—even though this may be but a small proportion of the total number of cells—confluent growth may occur on drug-containing medium obscuring the true resistant to sensitive ratio. A method based on enzyme induction would eliminate this problem since only the drug-resistant organisms could form the induced enzyme on drug medium while all cells would form the induced enzyme on the control medium. Overinoculation would only serve to reduce the time between inoculation and detection of the induced enzyme.

It should be emphasized that both adaptation and growth had taken place before sampling was started for growth and enzyme activity determinations.

Sampling was routinely started in these experiments when the adapted cell concentration reached about 10^6 cells per milliliter. The sensitivity of the present procedure is such that the presence of the induced enzyme can be detected from 3 to 5 generation times before growth can be detected by present optical means. It is possible that as techniques improve we may be able to detect the adaptive enzyme content of considerably fewer cells than are needed at present; this would make it possible to complete the test in an even shorter time.

While this method is obviously of little practical importance in testing the drug susceptibility of a fast-growing organism such as *Esch. coli*, it may assume clinical importance when one wishes to test the drug-susceptibility of a slow-growing organism such as *Mycobacterium tuberculosis*. Work is currently in progress to adapt this induced enzyme procedure to *M. tuberculosis* in the hope of developing a more accurate and rapid drug-susceptibility testing method.

SUMMARY

A new method has been devised which permits the quantitative determination of small numbers of streptomycin-resistant bacteria in the presence of streptomycin-sensitive cells. The method is based on the inhibition by streptomycin of β-D-galactosidase formation in sensitive cells, but not in streptomycin-resistant cells.

This project is based on a suggestion by Dr. Joshua Lederberg whose aid and interest in this work is greatly appreciated.

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EFFECT OF REUSE AND TEMPERATURE UPON RANA PIPIENS AS A PREGNANCY TEST ANIMAL

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SINCE the introduction of the use of *Rana pipiens* as a pregnancy test animal, consideration has been given to various factors which influence the test. These factors are variations in the test procedure,[1-6] maintenance of the test animals,[4, 7-11] seasonal variation in frog response,[3-5, 9-14] and sensitivity to chorionic gonadotrophic hormone.[8, 11-14]

Although Cutler[3] maintained that *R. pipiens* could not be used with reliability after a positive test, other amphibia have been used over again without loss of accuracy.[15] A standard procedure has been developed whereby animals used in one test could be reused a number of times. This report presents data obtained concerning the effect of reusing the test animals upon responsiveness. Information is also presented concerning the effect of environmental temperature.

METHODS

Maintenance of Frogs.—New frogs were placed in battery jars containing tap water and sulfadiazine. They were immediately placed in an illuminated refrigerator.[16] The frogs were removed as needed and placed in dry battery jars during the test. Upon completion of the test, tap water was added to the jar and kept at room temperature for varying periods of time. At the end of this period, fresh sulfadiazine was added and the frogs returned to the illuminated refrigerator until used again. Water was changed daily in the jars kept at room temperature, but only added when necessary to those jars in the refrigerator. No food was offered to the animals at any time. No check was made on the refractoriness of new frogs to chorionic gonadotrophic hormone (CGH) as this was accomplished during the course of the pregnancy test.

Urine.—Urine was concentrated by adsorption with kaolin. Fifty milliliters of urine was brought to a pH of 4.0 to 4.5 with 25 per cent HCl. Ten milliliters of a 5 per cent kaolin suspension was added and the mixture shaken. This was allowed to remain overnight in a refrigerator. Thirty to thirty-five milliliters of the supernatant was discarded and the remaining sediment and urine shaken and poured into a centrifuge tube. This was centrifuged for 10 minutes at 2,000 r.p.m. The supernatant was discarded, 5 ml. of 0.1 N NaOH was added, the mixture stirred and recentrifuged. The resulting supernatant was removed and made neutral with 25 per cent HCl. This procedure was given in detail in an earlier publication.[6]

Pregnancy Test.—Two frogs were used for each test and were taken from the same refrigerated jar. Two milliliters of the neutralized concentrate was injected into the dorsal lymph sac of each frog. Frogs were checked at 1- and 3-hour intervals after injection

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for the presence of sperm in their urine. Slides were examined under low power without a cover slip to insure motility of the sperm. The test was considered positive if at any time during the two examinations one or both frogs showed any motile sperm.

To minimize any errors due to refractory animals, frogs demonstrating a negative response after both checks were injected with 50 I.U. of CGH and checked again in one hour. Frogs showing no response to the CGH injection were considered refractory and discarded. Frogs were not checked for the presence of sperm prior to use since it was felt that any sperm from a previous test would have disappeared by the time of reuse. Since no false positive results were obtained, this assumption proved to be valid.[6]

RESULTS

The data obtained comparing the percentage of refractory animals with the number of times used are listed in Table I. Because of the acquisition of new frogs, it was necessary to eliminate older animals and, as a consequence, only 22 animals were utilized 5 times. The number of refractories obtained after the first test was equivalent to the percentage of nonresponsiveness in new animals, this being 5.2 per cent. Examination of the percentage of refractory animals indicated a definite increase in frequency of nonresponsiveness with increased usage.

TABLE I. EFFECT OF REUSE ON REFRACTORINESS OF RANA PIPIENS

NO. TIMES FROGS USED	NO. OF FROGS USED	REFRACTORY ANIMALS		χ^2
		NUMBER	PER CENT	
1	485	25	5.2	—*
2	251	27	10.8	7.07†
3	125	21	16.8	17.69†
4	79	13	16.5	12.06†
5	22	5	22.7	8.76†
Total	962	91	9.5	

*Used as first constant event.
†Significant at both the 1 and 5 per cent levels.

To determine the validity of these data, chi-square values were calculated from fourfold contingency tables. The data for new animals (used one time) were employed as the first constant event and Groups 2 through 5, respectively, as the second event. These values are recorded in Table I. Each value obtained is significant at both the 5 and 1 per cent levels. However, since the experimental variables were applied after the animals had been used the first time, the significance of the increase of refractoriness would have to be determined using Group 2 as the first constant event. Chi-square data were calculated using Group 2 as the constant first event and Groups 3 through 5 as the second event. The values obtained were as follows: Groups 2 and 3, 2.2; Groups 2 and 4, 1.3; and Groups 2 and 5, 1.8. None of these values are significant at the 5 per cent level.

To correlate some of the environmental factors which could have influenced the increase in refractoriness, data were accumulated concerning the effect of maintenance of animals at room temperature and refrigerator temperature. The results are shown in Table II. The significance of the percentage of refractory animals was determined by chi-square statistical analysis. The

figures in Table II were obtained for the animals kept at room temperature. A comparison of refractoriness was made for each day as compared to animals maintained at room temperature for 10 days. In a similar manner, the modal day 4 was selected as first event and all comparisons made against it for animals kept under refrigeration. Only one value in Table II proved to be significant at the 5 per cent level and that was for animals under refrigeration for only one day before reuse.

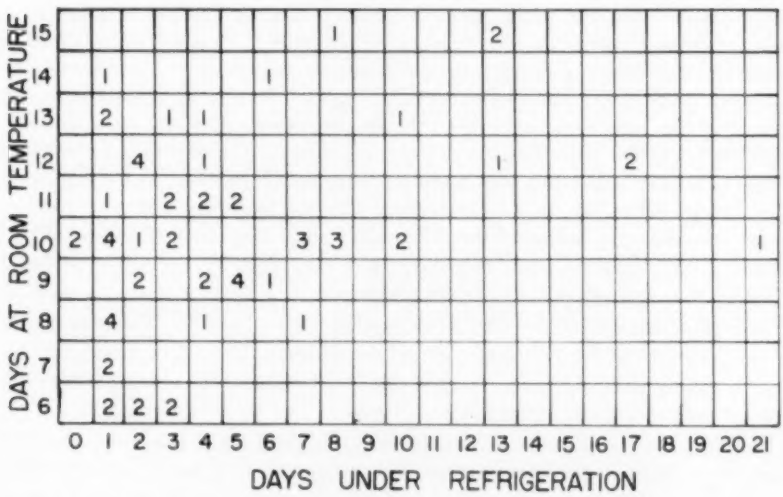

Fig. 1.—A composite of the effect of room temperature and refrigeration upon sensitivity. The number of refractory animals are shown for each combination.

TABLE II. EFFECT OF TEMPERATURE ON REFRACTORINESS IN RANA PIPIENS

ROOM TEMPERATURE			REFRIGERATION		
DAY	PER CENT REFRACTORY	χ^2	DAY	PER CENT REFRACTORY	χ^2
6	36.7	0.80	0	11.1	0.01
7	7.1	0.20	1	29.7	7.30*
8	18.2	0.00	2	22.5	2.60
9	15.7	0.00	3	11.6	0.01
10	15.5	—†	4	9.5	—†
11	10.2	0.00	5	23.1	1.80
12	15.2	0.02	6	6.7	0.00
13	12.2	0.10	7	10.5	0.02
14	7.7	0.60	8	10.2	0.01
15	18.8	0.04	10	13.6	0.02
			13	23.1	0.90
			17	15.4	0.02
			21	11.1	0.20

*Significant at the 5 per cent level.
†First constant event.

No simple statistical comparison was available for the combined action of the two environmental factors. Fig. 1 represents the number of refractory

animals found for the number of days under each environment as a composite. This figure showed a random distribution over the entire range, indicating no effect upon refractoriness when both environments are considered together.

DISCUSSION

The chi-square analyses upon the data in Table I confirmed the information shown by the increase in frequency of refractoriness of animals used 2 to 5 times. Since these data were significant at both the 5 and 1 per cent levels, it appeared that one or more of the experimental conditions to which the animals had been exposed did affect their reactive properties. To resolve the question of whether this significant increase in refractoriness was a function of the number of times the animals were used, chi-square data were calculated using Group 2 as the first constant event. Since the results obtained were not significant at even the 5 per cent level, the proportion of refractory animals in Groups 3, 4, and 5 was not significantly different from those in Group 2. It was concluded, therefore, that no significant increase in proportion of refractory animals occurred with increased use. The increase in frequency of refractoriness was due to some factor or factors occurring during or after the initial test and did not continue to affect the animals afterward.

It is interesting to note that the chi-square values in Table I do not follow the same pattern as the frequency of refractoriness. Instead of obtaining increasing values with increased usages, the high value was obtained between Groups 1 and 3, the rest being lower.

The fact that no significant results were obtained concerning the environmental effects indicated that these factors alone could not account for refractoriness. However, this information did influence the adoption of a definite procedure for the pregnancy test.

One of the factors which might have influenced the refractoriness of the frogs was the question of nutrition. This was not investigated during these studies, but loss in weight and general decline in appearance was noted in those animals used several times.

SUMMARY

Data were presented concerning the effect of reuse and environment upon the response of *Rana pipiens* to chorionic gonadotrophin. A significant increase in refractoriness was obtained after the animals had been used one time. No further increase was evident when frogs were used 3, 4, or 5 times. The length of time the frogs were maintained under different environmental temperatures did not affect the animals' sensitivity to CGH. These factors are discussed in relationship to refractoriness in frogs.

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DETERMINATION OF TOTAL LIPIDS, CHOLESTEROL, AND PHOSPHOLIPIDS IN CEREBROSPINAL FLUID

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KNOWLEDGE of the lipid content of cerebrospinal (CSP) fluid may contribute to the understanding of the changes occurring in demyelinating diseases. The myelin sheath consists mainly of lipoproteins. If, as a result of the demyelinating process, these lipids enter the blood stream, they would be difficult to measure because of the large and varying amount of blood lipids.[1] Radioactive tracer studies have shown that the uptake of substances from CSP fluid into the brain is rather rapid.[2, 3] If the reverse is true, and the lipid constituents of the myelin pass from the brain into the spinal fluid, then the changes due to diseases could be more easily detected in CSP fluid than in blood. No such investigations could be carried out in the past, however, because of the lack of suitable methods for the determination of lipids in the fluid. This paper describes procedures for estimation of total lipids, phospholipids, and cholesterol of CSP fluid.

PROCEDURES

Reagents for Extraction.—Chloroform-methanol (2:1).[4]

$CaCl_2$ Solution: this is prepared by shaking, in a separatory funnel, 96.3 ml. of chloroform-methanol (2:1) and 23.7 ml. $CaCl_2$ (20 mg./100 ml.). The two phases are allowed to separate and the upper phase is used.[5]

Total Lipids.—Chromic acid: Dissolve 20 Gm. of powdered $K_2Cr_2O_7$ in one liter of H_2SO_4 (sp. gr. 1.84).[6] Solution should be effected at 100° C.

Cholesterol.—$FeCl_3$ color reagent: dissolve 1.0 Gm. $FeCl_3 \cdot 6\ H_2O$ in 10 ml. of glacial acetic acid. Dilute 1.0 ml. to 100 ml. with concentrated H_2SO_4 and mix.[7]

Phospholipids.—Wet ashing mixture: Mix 32.5 ml. of 70 per cent HCl O_4 with 250 ml. of 20 N $H_2\ SO_4$ in a one liter volumetric flask. Dilute to the mark with H_2O.[8]

Ascorbic acid molybdate: 0.25 per cent ammonium molybdate in 0.1 N sodium acetate to which is added, just before use, 1.0 ml. of 10 per cent ascorbic acid per 10 ml. of solution.[8]

Extraction.—The extraction of lipids from cerebrospinal fluid is effected by adding 20 ml. of 2:1 chloroform-methanol to 10 ml. of fluid in a glass-stoppered test tube (22 by 150 mm.). The tightly stoppered tube is briefly shaken and immersed in a water bath at approximately 85 to 90° C. for several minutes. It is then shaken for an additional 2 minutes, during which time the emulsion is kept hot by reimmersion in the water bath. The tube

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is centrifuged for 20 minutes at 1,500 r.p.m. The upper phase, containing the water soluble constituents, is removed. Two milliliters of methanol is added with shaking, to the lower phase, and the extract filtered through fat-free Schleicher and Schuell "sharkskin" paper into a second glass-stoppered tube. The extraction tube is rinsed with 2 ml. of the same solvent mixture.

Purification of the Lipid Extract.—In order to remove traces of nonlipid material, the extract is shaken vigorously for 1 minute with 4 ml. of water. The tube is centrifuged for 20 minutes at 1,500 r.p.m. and the upper phase is discarded. To remove all traces of water, the walls of the tube are washed twice with 3 ml. of $CaCl_2$ solution; these washings are removed by suction and discarded.[5] The solvent is evaporated to dryness at 45 to 50° C. under a stream of nitrogen. The lipid residue is dissolved in 2 ml. of chloroform methanol and filtered into a 5 ml. glass-stoppered volumetric flask. The volume is brought to the mark with several rinses of solvent used to remove the last traces of lipid from the tube. An additional 0.50 ml. of solvent is then pipetted into the flask and the contents are thoroughly mixed by inversion. The extract is used for the following analyses.

Total Lipids.—The procedure for the determination of total lipids is based on the method of Bragdon.[6] A 2 ml. aliquot of the extract is pipetted into a test tube (16 by 150 mm.) that can be fitted with a glass stopper. The solution is evaporated to dryness under nitrogen at 45° C. and 1.0 ml. of chromic acid is added. The tube is stoppered and sealed with one drop of the reagent and then heated at 100° C. for 30 minutes. The tube is cooled in tap water and 4 ml. of distilled water is added. After 10 minutes, the optical density is read at 580 mμ. For the standard curve, serum lipids were prepared by the method of Sperry and Brand.[5] Quantities ranging up to 0.200 mg. were employed. The procedure would detect 10 μg.

Determination of Free and Ester Cholesterol.—The method of Hess[9] used for serum cholesterol, which is based upon adsorption on Al_2O_3 and selective elution followed by colorimetric estimation, is employed. The Liebermann-Burchardt color reaction, however, is replaced by that of Brown and associates.[7] The dry lipid extract of 10 ml. cerebrospinal fluid is dissolved in 5 ml. of petroleum ether, placed on an Al_2O_3 column (5 in. height, 0.5 in. diameter) and washed with 5 ml. of petrol ether. Cholesterol ester fraction is eluted by the addition of 20 ml. of 10 per cent ethyl ether in petroleum ether. The eluate, collected in a flask, is evaporated to dryness at 40° C. under a stream of nitrogen. The free cholesterol is eluted with 35 ml. of 10 per cent ethanol in petroleum ether. The eluate is taken to dryness as described for the ester. Both free and ester fractions are dissolved in 3 ml. glacial acetic acid; 2 ml. of ferric chloride sulfuric acid reagent is added and the color read at 560 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. The color is stable for 4 hours. If less than 10 ml. of spinal fluid is available, then it is not possible to separately determine both fractions; however, total cholesterol can be measured by the procedure of Brown and associates.[7] A 2.0 ml. aliquot of lipid extract is transferred to a 15 ml. glass-stoppered tube; the solvent is evaporated under nitrogen at 45° C. The residue is dissolved in 3.0 ml. of glacial acetic acid, and the colorimetric procedure described above is followed. For the preparation of the standard curve, 30 mg. of pure cholesterol is dissolved in 100 ml. of glacial acetic acid; aliquots up to 0.20 ml. are employed. The procedure would detect 10 μg of cholesterol.

Phospholipids.—Phospholipids are determined by a modification of the micromethod of Lowry and associates.[8] The solvent is evaporated from the aliquot of lipid extract, and 0.75 ml. of wet-ashing mixture is added to the residue. The sample is heated at 95° C. for 2 hours and at 165° C. for 2 hours. After cooling, 4.5 ml. of ascorbic acid-molybdate mixture are added. The optical density is read at 820 mμ. For the standard curve, 0.4 mM. KH_2PO_4 was used in volumes ranging from 0.1 to 1.0 ml. The appropriate blank[8] consists of an equal volume of the solvent mixture treated in the same manner as the test solution. The phospholipid content was calculated by multiplying the phosphorus values by a factor of 26. As little as 0.15 μg of lipid phosphorus could be measured.

RESULTS

Total Lipids.—Because of the low protein concentration in CSP fluid, the lipids that are conjugated with these proteins might be expected to be proportionally low. Consequently the methods used for blood lipids cannot be applied directly to fluids. The procedure of Sperry and Brand[5] uses a serum-solvent ratio of 1:25 for lipid extraction. Because of the low concentration of lipids in the fluid, it is necessary to use about 10 ml. for extraction. This would necessitate 250 ml. of solvent—a volume which is impractical for an analytical procedure. Therefore, the volume of the solvent had to be reduced. Various ratios of solvent to fluid were tried. Aliquots ranging from 2.5 to 15.0 ml. were extracted with 20 ml. of 2:1 chloroform-methanol and analyzed for total lipids as described above. As the basis for comparison, 10.0 ml. of the same fluid was concentrated to 1 ml. under N_2 at 45° C. and the total lipids extracted by the method of Sperry and Brand,[5] using their ratio of 1:25. The amount of lipid in the concentrate was determined by both gravimetric and chromic acid analyses. Values of 2.50 and 2.45 mg. per cent, respectively, were obtained. The results show (Table I) that the recovery of total lipids from the fluid ranged from 98 to 101 per cent. In the range studied, variation in the fluid to solvent ratio had no effect on the extractability of the lipids. The ratio of 10 ml. of fluid to 20 ml. of solvent was adopted because this volume of fluid is readily obtained by spinal tap and provides enough material for the analysis of total lipids, phospholipids, and cholesterol.

TABLE I. INFLUENCE OF SPINAL FLUID-SOLVENT RATIO ON THE EXTRACTION OF LIPIDS

ML. OF CSP FLUID	RATIO OF FLUID TO SOLVENT	MG. LIPID		PER CENT RECOVERED
		FOUND	CALCULATED	
2.5	1:8	0.062	0.062	100
5.0	1:4	0.123	0.124	99
10.0	1:2	0.249	0.248	101
15.0	1:1.5	0.362	0.372	98

TABLE II. TOTAL LIPID CONTENT OF DILUTED SERUM

SERUM DILUTIONS	MG./10 ML.	VALUES BASED ON UNDILUTED SERUM* MG.	PER CENT RECOVERED
1:25	2.328	2.320	100
1:50	1.160	1.160	100
1:100	0.560	0.580	97
1:200	0.288	0.290	99

*The total lipid concentration of the undiluted serum was 580 mg. per cent.[5]

The recovery of lipids from spinal fluid cannot be tested by the usual procedure of adding a measured quantity of the solid compound because lipids added to fluid do not go into solution. Therefore, blood sera was diluted twenty-five to two hundred fold to simulate spinal fluid concentrations. Total lipids were determined on extracts of those diluends. The lipid concentration of the undiluted serum was determined according to Sperry's gravimetric method, and this value was used as a basis for calculating the recovery. The recovery of lipids,

as can be seen in Table II, was between 97 and 100 per cent. The reproducibility of the method was determined by analyzing 6 replicates; the average value was 2.4 mg. per 100 ml. with a standard deviation of ± 0.027.

Cholesterol.—The extractability of cholesterol was tested by comparison of our procedure with the original method of Brown and associates.[7] Spinal fluid was concentrated in a 50 ml. volumetric flask from 10 to 2 ml. with a stream of nitrogen (45° C.). As a blank, 10 ml. of 0.9 per cent NaCl was used. Thirty milliliters of alcohol-acetone (1:1) was added to the concentrated fluid, and the mixture was brought to boiling in a water bath. Because NaCl was found to react with the $FeCl_3$ color reagent, it was necessary to remove it from the alcoholacetone filtrate. A 10.0 ml. aliquot of the filtrate was evaporated to dryness in a test tube and the residue dissolved in 5 ml. of chloroform methanol (2:1). The extract was shaken vigorously with 1 ml. of H_2O, centrifuged, and the lower phase used for cholesterol analysis. Aliquots of 5.0 and 10.0 ml. of the same fluid were extracted as outlined and the extracts analyzed for cholesterol. The average of 4 replicate determinations on the 5 ml. aliquots was 0.40 mg. per 100 ml. and on the 10 ml. aliquots, 0.39 mg. The amount found by the method of Brown[7] was 0.41 mg., indicating recoveries of 98 and 95 per cent, respectively. The method is sensitive to 10 μg of cholesterol.

In order to test the efficiency of the method of separation of free from ester cholesterol, aliquots of solutions containing equal amounts of the two in concentrations of 20, 30, and 40 mg. per 100 ml. were chromatographed. The recoveries of free and ester cholesterol were 97.7 and 98.0 per cent, respectively. The method was applied to blood sera diluted 100 times. Three samples were analyzed in triplicate; the average amount of total cholesterol found, calculated on the original serum, was 164.9 mg. per 100 ml. The per cent of free cholesterol was 31.7—a value comparable to that found by Hess[9] in a series of normal sera.

TABLE III. CHOLESTEROL IN SPINAL FLUID

FREE (MG./100 ML.)	ESTER (MG./100 ML.)	FREE (PER CENT)
0.189	0.214	46.8
0.351	0.417	45.7
0.344	0.209	62.2
0.385	0.547	41.3
0.324	0.209	60.8
0.278	0.131	68.0
0.389	0.248	61.0
0.262	0.180	59.4

Recovery studies were also conducted by adding to the diluted serum 20 mg. each of both free cholesterol and the equivalent of cholesterol stearate. The average recoveries on 4 replicate determinations was 105 per cent for both the free and ester cholesterol. The method was then applied to a series of spinal fluids; the results are given in Table III. Many of the fluids analyzed were from various neurologic disorders, but the results are not being correlated with any pathology at this time. It might be noted that the concentrations of free cholesterol are below the amount that can be precipitated quantitatively by digitonin.[10]

Phospholipids.—The extractability of phospholipids has been tested using various volumes of fluid. The fluids were extracted with 20 ml. of chloroform-methanol and analyzed for lipid phosphorus as outlined. An aliquot of each fluid was lyophilized, the residue suspended in 1 ml. of water and extracted with 25 ml. of chloroform-methanol. The value obtained from the lyophilized fluid was used as the basis for comparison. The results, presented in Table IV, show that the recovery from the lyophilized fluid was complete. Parallel experiments on serum diluted two-hundred fold produced an average phospholipid recovery of 101 per cent. The reproducibility of the method was determined by analyzing 6 replicates, the average value was 1.33 mg. per 100 ml. with a standard deviation of ± 0.10.

TABLE IV. EXTRACTABILITY OF PHOSPHOLIPIDS FROM CEREBROSPINAL FLUID

ML. OF SPINAL FLUID	MG. OF PHOSPHOLIPIDS		PER CENT EXTRACTED
	FOUND	EXPECTED	
2.5	0.02026	0.02057	98.2
5.0	0.04056	0.04114	98.5
10.0	0.08215	0.08229	99.8

SUMMARY

Analytical methods are given for the determination of total lipids, free and ester cholesterol, and phospholipids in spinal fluid.

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KELL TYPING IN THE CAPILLARY TUBE

An Adaptation of Löw's Papainized Serum Technique to the Capillary Method of Blood Grouping

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NEXT to the antigens of the Rh system, Kell plays the most important role as a cause of hemolytic disease of the fetus and the newborn infant and of transfusion reactions.* Sensitization to this factor is usually induced by transfusion, although it may result from stimulation by pregnancy alone; in our experience if the mother has been sensitized by transfusion the resulting hemolytic disease due to anti-K tends to be severe, not uncommonly resulting in stillbirth or hydrops. Is the risk of sensitization to Kell, and, therefore, the threat of disease or of reaction, of sufficient magnitude to warrant testing of donor and patient with anti-K, or is the task of such testing too great for the blood bank to assume?

This question has been raised many times, and, while all agree that such sensitization is undesirable and is to be avoided if possible, the time involved—the standard method being the indirect Coombs' technique—the cost, and the amount of serum that would be required have been held to be prohibitive. The method here described overcomes these objections. It is accurate and is economical of both time and serum; in less than one hour an experienced technician can test 200 blood samples, check the Kell-positives and record all the results. The expenditure of serum depends upon its strength. In our first test-run of 15,000 donor specimens we used an undiluted anti-K serum with a titer of 1:128 determined by both indirect Coombs' and papain methods. This gave us 300 to 350 tests per milliliter. Later we found a dilution of 1 part of this anti-K serum to 20 parts of AB serum to be quite satisfactory, although the reactions were somewhat slower. This dilution allowed a minimum of 6,000 tests per milliliter of neat serum.

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Based on a paper read at the tenth Anniversary Meeting, American Association of Blood Banks, Chicago, Nov. 4-6, 1957.

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*Dr. Eloise Giblett has kindly allowed us to quote the following figures from her paper, "The Incidence of Red Cell Antibodies and the Consequent Danger of Transfusion in a Community," read at the tenth Anniversary Meeting of the American Association of Blood Banks in Chicago, Nov. 4-6, 1957. Of 21,000 recipients 267 were found to have antibodies other than anti-A or B. Of these, 194 were antibodies of the Rh system alone. Of the remaining 73, 40 were antibodies not likely to cause either hemolytic disease or severe transfusion reaction, i.e., anti-Le^a, Le^b, M, or P. Of the remaining 33 potentially dangerous antibodies there were 25 examples of anti-K, 3 anti-K + anti-D, 1 anti-K + anti-E, 1 anti-K + anti-Fy^a. In other words 30 of the 33 potentially dangerous antibodies outside the Rh system were anti-K alone or in combination with another antibody.

MATERIALS

Anti-K Serum.—Test your neat serum; if there is a good reaction in less than 10 minutes then set up serial dilutions of the serum in AB serum and test each dilution by the method. Select for use the dilution that gives a good reaction within a decided time limit; we like to use a dilution that reacts within 10 minutes. Commercial sera are practically always diluted; they are satisfactory for use by this method *only* if the diluent is AB serum. Our serum is dispensed in serology tubes containing 0.25 to 0.5 ml. and is kept frozen when not in use.

Papain Solution.—We used the German brand of papain kindly supplied us by Dr. Löw* and made up according to his instructions.[1] We understand that Merck's American papain does not work in the tube, but we have had no experience with it. The solution is first put up in 5 to 10 ml. units and frozen. One lot at a time is thawed and redispensed in units of 0.25 to 0.5 ml. in serologic tubes (55 by 6 mm. outside diameter) and again frozen. When papain is thawed for use it is mixed well; it can then be allowed to settle and the supernatant used, although this is not necessary as the inactive sediment does not interfere with the reaction. The papain keeps indefinitely if kept frozen.

Capillaries.—The capillaries are 9 cm. long by 0.4 mm. internal diameter—the so-called "coagulation tubes." We have found those supplied by Progressive Laboratory Specialties Co., Jamaica, N. Y., uniformly satisfactory.

Slant Box.—The type we have used for the past 12 years is illustrated in Fig. 1; any handyman can make it. The lights are to be turned on only for reading the reactions, *not* for heat.

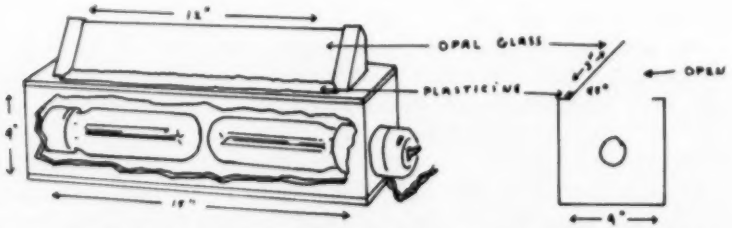

Fig. 1.—The slant box seen from the front and endwise. The opal glass is pure white or a bluish white. The light is turned on to facilitate reading *not* for heat; consequently it is turned on only when making readings.

Blood Samples.—Pipette free cells from the clots into properly numbered test tubes. (To save washing glass pipettes we use paper straws for this purpose.) Fill the tubes with saline; centrifuge. Throw off the saline with a quick flick, leaving a cell suspension of approximately 50 per cent and place the samples in order in a rack. Samples we have tested for the Red Cross have been pilot tube specimens with which they were finished. Saline had already been added, and by the time we received them the cells were partially settled; we threw off the supernatant saline and proceeded without further washing. In our experience the Kell antigen does *not* become nonreactive quickly after blood is drawn, as is often stated; Kell-positive cells a week old will react well if freshly washed.

METHODS

1. We like to work in units of 10; our racks carry 4 rows of 10 tubes each. Shake the rack of cell suspensions well and place it in front of the slant box. On the bench in front of the rack place a paper towel, folded as it comes from the dispenser.

*Papayotin, 1:350; Merck.

2. Place a number of capillaries between the second and third fingers and the tube containing the anti-K serum between the thumb and first finger of the left hand.

3. With the right hand take a capillary from the left hand and dip the tip into the tube of anti-K serum; allow about ½ inch of serum to run in. Lean the capillary on the rack, the empty end resting on the edge of the rack, the serum-filled end on the paper towel. Repeat with the needed number of capillaries. It is preferable to dip only one capillary at a time into the serum; otherwise serum runs between the capillaries and is wasted.

4. Place the small tube of papain solution in the crook of the little finger of the left hand. With the thumb and first finger of the left hand take the first blood sample. Pick up a serum-loaded capillary and dip the serum-containing end into the tube of papain solution allowing approximately twice as much papain solution to run in as the column of serum already in the capillary. Wipe the capillary with remaining fingers, and dip the serum-papain-filled end of the capillary into the cell suspension allowing ¼ to ½ inch of the cell suspension to run in.

5. Invert the capillary; place the forefinger over the upper end to prevent the contents from running out and set the capillary against the slanted glass with its lower end in the plasticine.

6. Replace the first tube of blood in the rack and with the same movement pick up the second tube of blood. Pick up a second capillary containing serum, dip it in the papain solution, then in the blood, invert and set up. Repeat for each blood sample. The capillaries are set up in groups of 10, representing the rows in the rack. Capillaries for samples 1 to 10 are set close together, then a space left and capillaries for 11 to 20 set close together, and so on.

RESULTS

The time from setting up to visible reaction will depend upon the serum used. As we said before we like to work with a serum or AB dilution of serum that gives a reaction within 10 minutes.

Kell-Positives.—The reaction of K-positive samples starts with a curdling appearance of the bottom of the blood column as it settles in the capillary. As the cells flow down and settle to form a butt at the lowest point this "curdling" is obliterated and crossbars start to form the length of the blood column. These crossbars finally aggregate into relatively large clumps with clear fluid between the clumps. After some time if the reaction is particularly strong, these clumps may all slide down to rest on the butt of cells, leaving a column of clear fluid.

Kell-Negatives.—The cells of the K-negatives also form a butt at the bottom of the capillary contents. Above this they recede from the lateral walls of the capillary to form a smooth needlelike column of cells the length of the capillary. The column will remain unbroken for hours.

Precautions.—

Check of Kell-positive bloods: At the end of the run remove the tubes of cell suspensions that you have recorded as positive, placing them in a rack. Repeat the test on these. This serves as a check on the original reaction and also on your recording, e.g., that it is in fact tube 4 of row 2 and not tube 5

of row 2 that reacted. In the beginning, and until you have gained confidence through experience in the method, you may well check these same bloods by the indirect Coombs' method.

Direct Coombs'-positive samples: Cells that are Coombs'-positive will give a positive reaction with any serum whatsoever, inert or other, to which papain has been added.

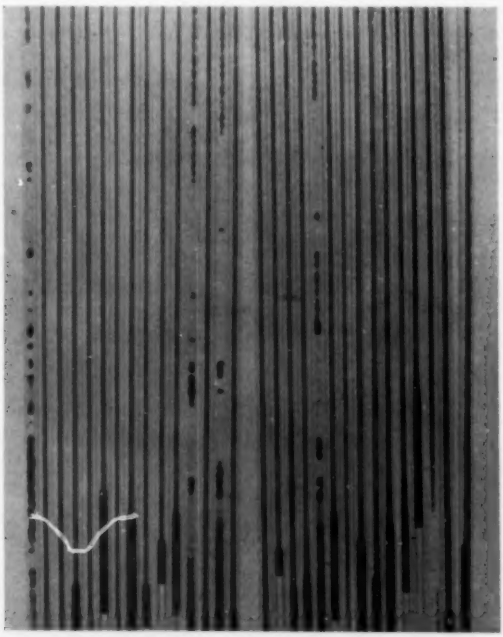

Fig. 2.—Examples of positive and negative reactions at 10 minutes. Numbers 1, 12, 14, and 20 are positive. In this illustration the bloods are set up in groups of 15 because that was the way the Red Cross bloods referred to in the script came to us. In our usual routine they are set up in groups of 10.

Contamination of papain solution: Once a tube of papain is thawed it must be used for *one* serum and *one* serum *only*; the residue is discarded at the end of a day's run with *that* serum. As the serum-charged capillaries are dipped into it it becomes slightly but progressively more contaminated with that serum.

Air bubbles preventing mixture of contents: If you pick up an air bubble between any two units of the reacting mixture—between serum and papain or between papain and red cells—the contents are blown out on a fingernail and allowed to flow back in. Until you become expert in the manipulation of capillaries you might find it less bother to discard a capillary in which you

have picked up an air bubble. Air bubbles can be avoided by not loading too many capillaries with serum at a time until you become quick at setting them up.

Occasionally a K-positive reaction is so strong that a single great clump clings to the lateral walls the whole length of the capillary; check such an apparent reaction with a less heavy cell suspension.

DISCUSSION

This method was first incorporated in our routine techniques as a quick way of detecting some incomplete antibodies, and later as a method allowing us to use incomplete anti-c, anti-E, and anti-K in the capillary. Any saline antibody will react in the capillary without the addition of papain[2]; however, where the antibody is weak the reaction can be considerably enhanced by the use of papain. We have tested 40 examples of anti-K by this method; all worked, but naturally some were quicker than others.

In a period of 4 months we tested 15,000 donors for the Manitoba Division of the Canadian Red Cross Blood Transfusion Service through the cooperation of Dr. Lyonel Israels and Miss Catherine Anderson. The K+ frequency was 8.34 per cent, which does not differ significantly from previous series we have run by the indirect Coombs' method. The first 500 samples were run in parallel by the present and the indirect Coombs' method with complete agreement. A further 200, negative by the present method, were checked by indirect Coombs' and were negative by that method. In the 15,000 there were 12 samples which showed slow, weak agglutination causing some doubt in reading. Four of these were positive by the direct Coombs' method. The other 8 occurred early in the series and were the result of cell suspensions that were too thin; when checked with a heavier cell suspension, clear-cut results were obtained which agreed with indirect Coombs' checks on those samples.

Kell-positive donors need not be lost to the blood bank; their blood can be used with discrimination (1) for transfusion of Kell-positive patients; (2) for transfusion of elderly patients unlikely to require further transfusions; and (3) for plasma. However, we think that all Kell homozygous (KK) donors should be set aside to be part of a special panel. Their blood should be completely typed by a reference laboratory so that, should a patient with anti-k (Cellano) in her serum, with or without other antibodies, need transfusion, the required blood may be available.

SUMMARY

By adding Löw's activated papain solution to anti-Kell serum in a capillary and immediately taking up test cells a clear distinction between Kell-positive and Kell-negative cells can be made. This method allows an experienced technician to test 200 bloods an hour, with an expenditure of 1 ml. of serum per 300 to 6,000 or more tests, depending upon the strength of the serum. The

blood of Kell-positive donors may be used for transfusion of certain patients or for plasma production. Homozygous Kell-positive donors should be kept in reserve to be called upon for special cases.

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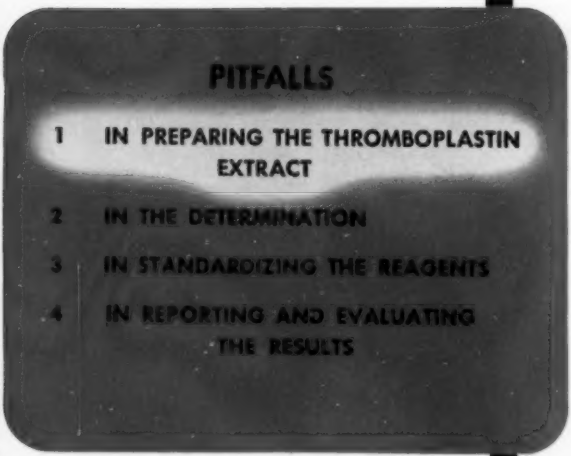
2. Chown, B., and Lewis, M.: The Slanted Capillary Method of Rhesus Blood-grouping, J. Clin. Path. 4: 464-468, 1951.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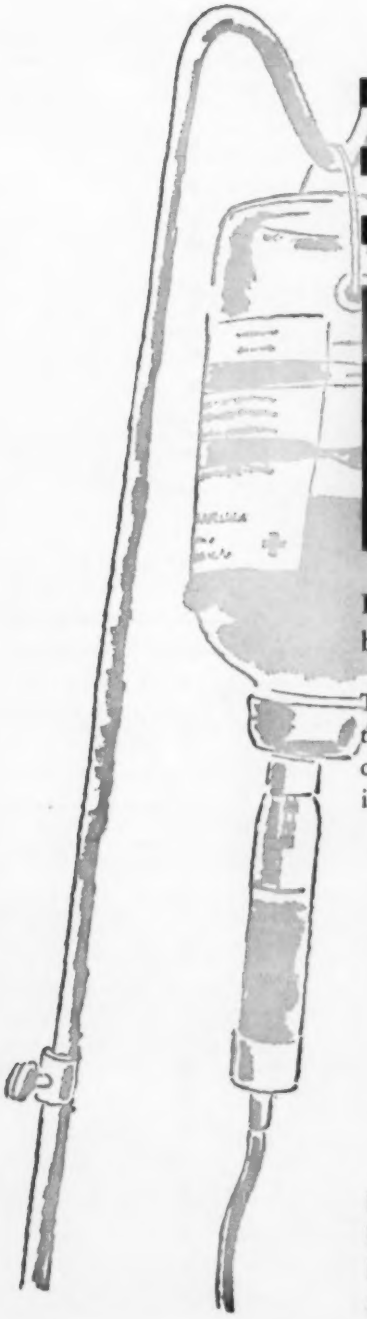

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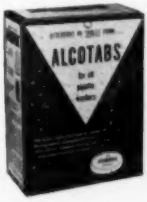

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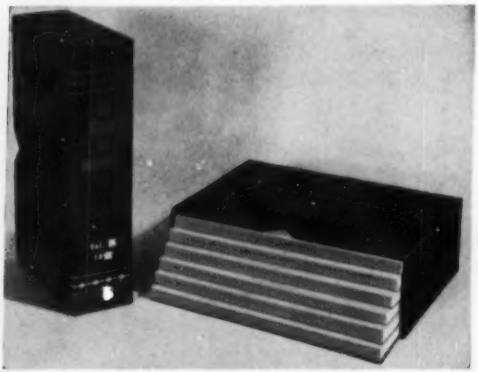

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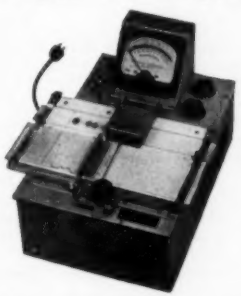

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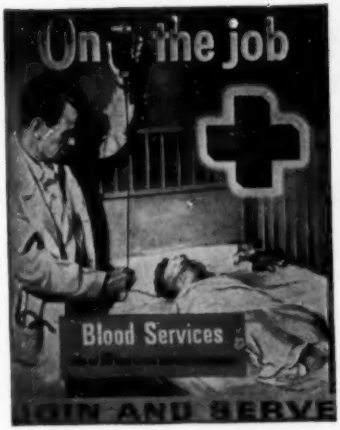

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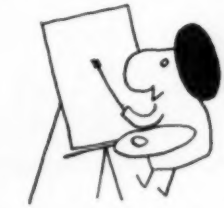

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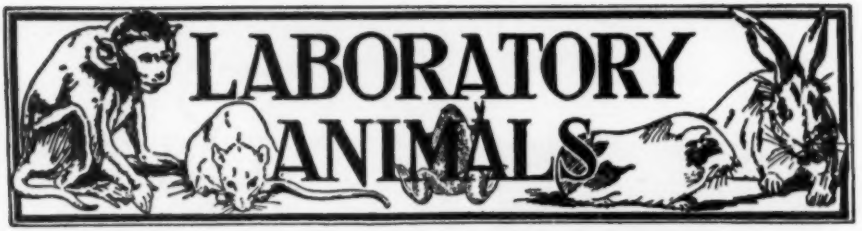

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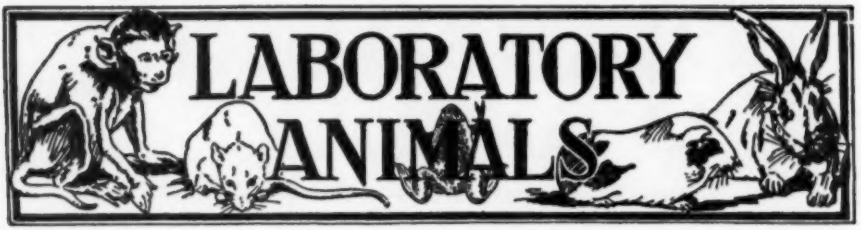

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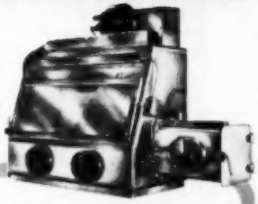

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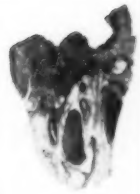

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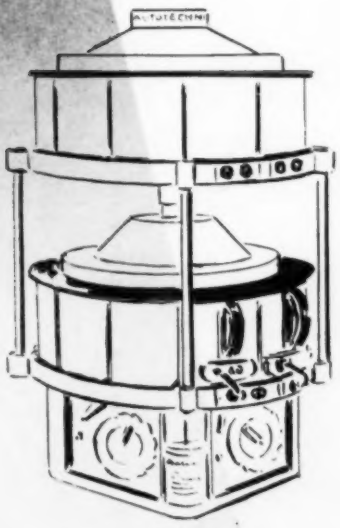

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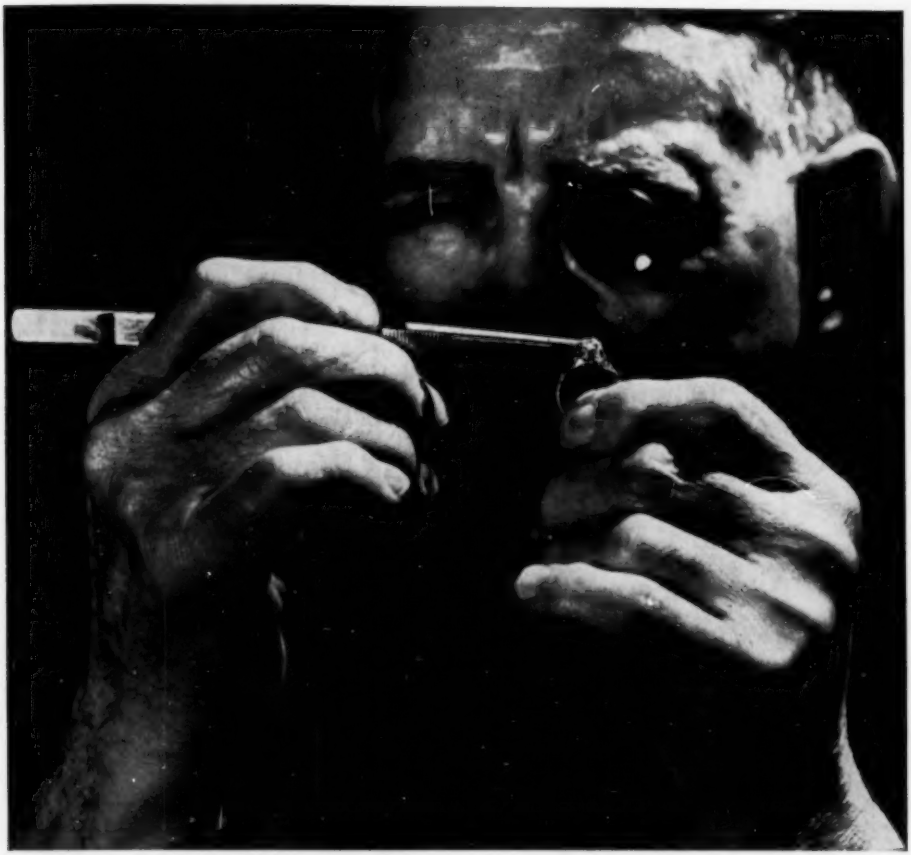

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